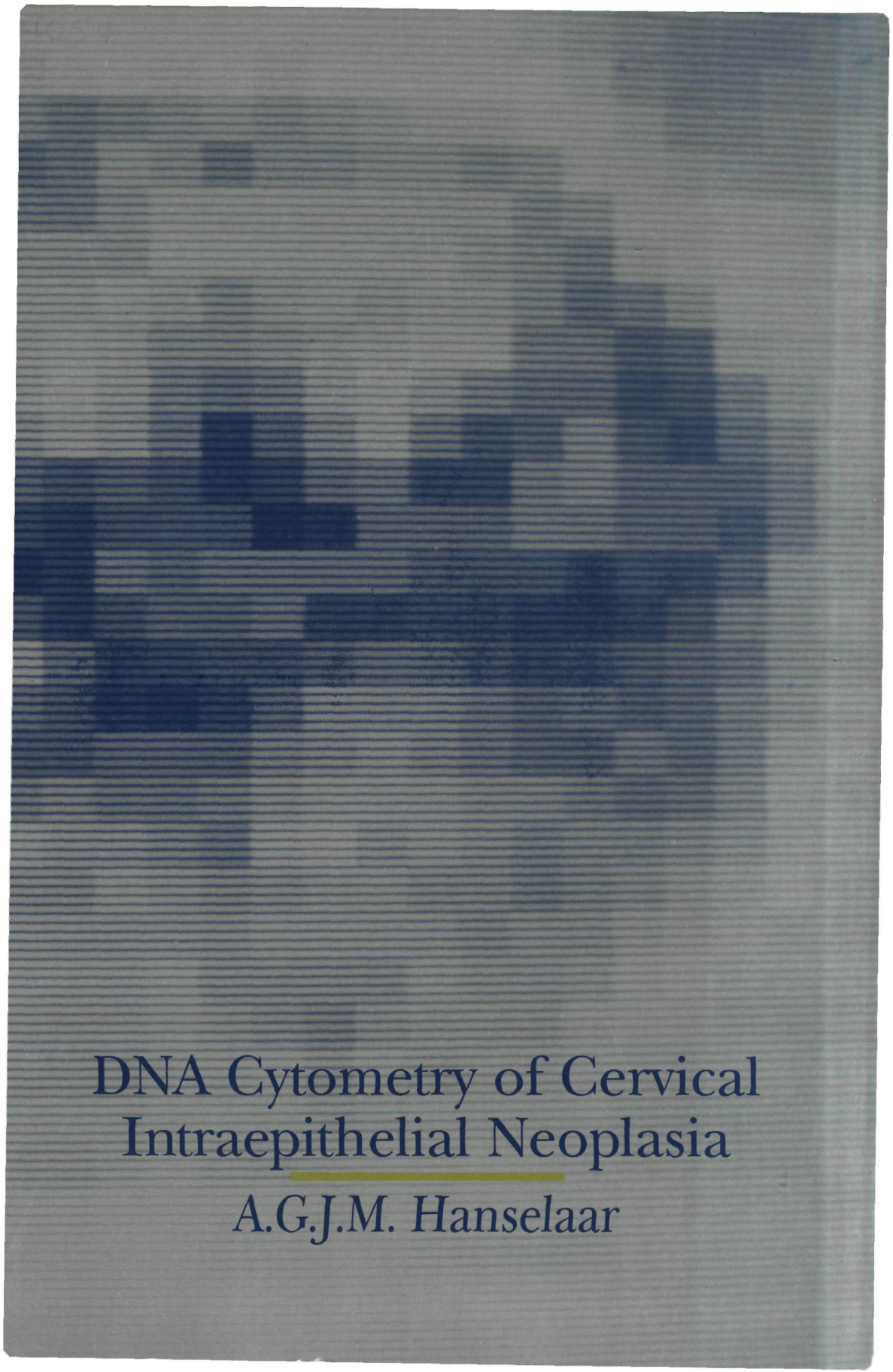

DNA Cytometry of Cervical Intraepithelial Neoplasia

A.G.J.M. Hanselaar

DNA CYTOMETRY OF

CERVICAL INTRAEPITHELIAL NEOPLASIA

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op het gebied van de geneeskunde en tandheelkunde,
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Cover: Photograph of a digitized image of an interphase cell nucleus, stained with a DNA specific Thionin-Feulgen stain. Styling: Eugene Arts.

CONTENTS

ABBREVIATIONS

ADI	Average DNA Index
AREA	2-D Area of the nucleus
CIN	Cervical Intraepithelial Neoplasia
CIN 1-3	Cervical Intraepithelial Neoplasia Grade 1-3
CIN.INV	CIN III adjacent to INV
CIRC	Circularity
CONTR	Contrast
CPM	Cytophotometry
CS	Cervical cytological smear
DI	DNA Index
DMOM1	First Diagonal Moment
DMOM2	Second Diagonal Moment
DNA	Desoxyribose Nucleic Acid
ENTRO	Entropy
FCM	Flow Cytometry
GLD	Greylevel Distribution
GMRAT	Geometrical Moments Ratio
HOMOG	Homogeneity
HPV	Human Papilloma Virus
ICM	Image Cytometry
INV	Invasive Squamous Cell Carcinoma
INVCON	Inverse Contrast
IOD	Integrated Optical Density
LRL	Long Runlength Emphasis
MASSEC	Mass Eccentricity; DNA margination
M3DI	Third Moment of DNA Index
MXTRP	Maximum Transition Probability
NIAS	Nijmegen Image Analysis System
PEC	Cytospin specimen, retrieved from paraffin-embedded tissue
RLD	Runlength Distribution
RPC	Run Percentage
SDDI	Standard Deviation of DNA Index
SRL	Short Runlength Emphasis
TRISY	Triangular Symmetry

CHAPTER 1

INTRODUCTION

Since the introduction of cervical cytological sampling for the diagnosis of cancer and premalignant lesions, the surface of the uterine cervix has become one of the most extensively studied areas (56,75). As a consequence of these efforts it is now generally accepted that invasive squamous cell carcinoma of the uterine cervix develops through a continuum of intraepithelial changes representing stages of malignant transformation of the cervical mucosa. By recognizing intraepithelial neoplastic changes, a potentially malignant transformation can be identified in an early phase before signs or symptoms have occurred, although many epithelial abnormalities may mimic true progressive changes. Therefore during the last decades many studies have concentrated on the morphological or functional identification of true progressive intraepithelial abnormalities.

The uterine cervix is located in such a way, that cytological, histological and colposcopical examination is relatively simple. The surface of the uterine cervix is covered by several layers of squamous cells on the ectocervix and by a single layer of columnar epithelium in the endocervical canal. These two types of epithelium meet at the so called squamocolumnar junction. During adolescence this junction is turned outward and as a consequence a relatively large area of columnar epithelium, the transformation zone, is exposed to the vagina. In response to the acidity of the vaginal environment the columnar epithelium may undergo squamous metaplasia, passing through the phases of reserve cell hyperplasia and immature squamous metaplasia. In this area dedifferentiation of the epithelium may occur leading to the development of invasive squamous cell carcinoma. These epithelial changes have rather arbitrarily been subdivided into slight dysplasia (= Cervical Intraepithelial Neoplasia grade 1 = CIN1), moderate dysplasia (CIN II) and severe dysplasia and Carcinoma in Situ (CIN III). The histopathological subdivision is paralleled by an increasing atypia, irregular arrangement of the cells and increased mitotic activity.

Despite the large amount of cytological and epidemiological data that has become available, the cellular biology of premalignant cervical lesions has not yet been solved. There are considerable differences in the results of follow-up studies, depending on issues like different interpretation of cytological and histological features and different statistical approaches of epidemiological data (10,20,26,35, 38,52,58,59,64,70,76,78,82,84,95). A study that often has been referred to is the study of Richart and Barron, who performed a follow-up study of 557 women, with abnormal smears, the most severe lesion of these being moderate dysplasia (CIN II) (84). Patients were followed by cytological and colposcopical examinations without interference of punch biopsies or other treatment. The average follow-up period was 36 months. Using a statistical model they calculated that in a 10 year period moderate dysplasia would progress to a more severe lesion in about 90 % of cases. Patients were admitted only after three (successive) abnormal smears.

In table 1 an overview of some of the literature on the natural history of cervical intraepithelial neoplasia is presented in more detail.

Table 1: Overview of some of the literature on the natural history of cervical intraepithelial neoplasia.

	N of Pat.	Select.* Criter.	Initial Diagnosis	Regr.	Persist.	Progr.	Progr. (CIN3)	Follow-up (INV)period
Nasiell	411	C	CIN2	50%	15%	35%	0.3%	50-78 mon.
Hall	85	B+C	CIN2	33%	49%	18%	-	1-14 yrs
Richart	557	C/3xdys.	dysplasia	6%	-	94%	-	36 months
Fox	278	C	CIN1+2	31%	9%	59%	1%	15-40 mon.
	33	C	CIN3	R+P	100%	-	-	
Koss	26	B+C	CIN1+2	39%	15%	42%	4%	1/2-7 yrs
	67	B+C	CIS	25%	61%	-	6%(13%)	
Villa S.	63	B+C	dysplasia	48%	16%	16%	21%	0-8 yrs
Kottmeier	31	B+C	CIS	-	-	-	71%	>12 yrs
Petersen	127	B+C	atypia	-	-	-	27%	3-16 yrs
Kinlen	52	C	Pap4-5	37%	38%	-	25%	>2 yrs
Green	576	B+C	CIS				0.17%	1-12 yrs
Mcindoe	131	B+C	CIS	8%	69%	-	22%	1-28 yrs

* B+C: Cytologic follow up after initial histological diagnosis, both representing CIN lesions.
C: Cytologically followed, no histological examination prior to treatment.
Mild and moderate dysplasia are referred to as CIN I and II, respectively.

Many reports of follow-up studies dealt with CIN I and II lesions. Data on the follow-up of CIN III lesions are less frequent. Some studies were based on cytological follow-up alone, others on cytological examination after initial biopsies or conisations. Nasiell et al followed cytologically 894 women with moderate dysplasia (CIN II) (70). Patients were entered into the study after one smear indicating moderate dysplasia. Biopsies were performed in 483 (54 %) women. Results of 411 cases without biopsy were: regression in 50 %, persistence in 15 % and progression in 35 %. Invasive carcinoma occurred in three patients (0.3 %). The average follow-up period was 78 months for cases that showed regression, 50 months for cases that showed persistence and 51 months for cases that showed progression. In a study of Hall and Walton 85 cases of moderate dysplasia were followed by cytological and histological examination from one to 14 years (38). The regression rate was 33%, persistence 49% and progression to carcinoma in situ in 18%. No invasive carcinoma was seen. In a study of 278 patients with initial cytological diagnosis of mild or moderate dysplasia (CIN I and II), Fox found percentages for regression and persistence of 31 and 8.9 respectively (27). The reported progression to severe dysplasia and carcinoma in situ (CIN III) was 59 % and to invasive squamous cell carcinoma 1.1 %. Most of these lesions were undisturbed by tissue study. In his study, Fox also reported follow-up results of 33 cases of severe dysplasia and carcinoma in situ, that were diagnosed and followed by cytological examination alone. In this series no invasive carcinoma occurred (27). The follow-up period was 15 to 40 months. In an early paper, Koss reported a group of 26 women with "borderline" lesions of the cervix, comprising mild and moderate dysplasia (CIN I and II) (58). Regression was seen in 39 %, persistence in 15 %, progression to CIN III in 46 % and to invasive carcinoma in 4 %. Initial diagnoses were established by biopsy. Follow-up was by cytological examination only. The follow-up period was seven months to 10 years. Koss et al further reported follow-up data of a group of 67 women with carcinoma in situ of the cervix, initially diagnosed by biopsy. Four cases progressed to invasive cancer (6%) and five cases developed questionable invasion (7%) (58). Regression was present in 25 % and persistence in 61 %. Kottmeier reported in 1961 that 22 (71%) patients of a group of 31 patients with CIS, who had been followed for at least 12 years, had developed invasive carcinoma (59). Petersen reported follow-up data of a group of 127 patients with epithelial atypia, who developed invasive cancer in 27% of cases (78). Kinlen and Spriggs presented follow-up data of a group of 131 women with a cytological diagnosis of unsuspected positive smear

(Papanicolaou Class IV or V) who had been lost to follow-up for at least two years (52). They traced 52 women, with positive cervical smears at least two years previously but who were not treated and had had no biopsies. Thirteen (25 %) of these women had developed invasive carcinoma. Nineteen (37%) had no apparent abnormalities when reexamined. In 20 patients (38%) at follow up CIS or dysplasia was diagnosed. Further studies were carried out in the National Women's Hospital, Auckland, New Zealand by Green and others (35,64). Initially Green and Donovan found a progression rate to invasive cancer in patients with cervical carcinoma in situ, diagnosed by cone biopsy, of 0.17%. One of their statements was that either the length of the preinvasive phase of cervical squamous cell carcinoma is much longer than 20 years or very few in situ cancer ever become invasive (35). Mcindoe et al from the same hospital, presented in 1984 an overview of follow-up data of 948 patients with carcinoma in situ, initially diagnosed by biopsy or conization, followed from five to 28 years (64). One subgroup of 131 patients continued to produce abnormal cytology consistent with cervical extra epithelial neoplasia of which 29 (22%) developed invasive carcinoma of the cervix or vaginal vault.

All these studies have been criticized in one way or another. Regarding technical aspects, it has been argued that if the initial diagnosis was made at histological examination, the lesion itself may have been removed or altered in such a way that regression of the lesion was promoted (58,84). On the other hand cytological examination alone has been considered to be inaccurate at diagnosing the earliest stages of cervical invasion. Concerning ethical aspect of these studies, it has become clear that once a risk of progression from carcinoma in situ to invasive cancer is accepted, it is ethically not acceptable not to treat patients at the carcinoma in situ stage (26,77).

These studies have shown that CIN lesions indeed may progress into invasive cancer. Furthermore it has become clear that CIN I as well as CIN III lesions may progress to invasive cancer (58,84). A significant part of the CIN lesions when left untreated will however not have a progressive course. The precise frequency of progression of CIN into invasive cancer still remains unknown. Koss recently stated in an overview article that the ratio of precancerous lesions of the uterine cervix to invasive cancer is probably of the order of 10 to 1, possibly even higher, and that as such, at most one in ten precancerous lesions is likely to progress to invasive cancer, when left untreated (56).

But whatever the precise percentage of progression or non-progression of CIN may be, light microscopically CIN I-III lesions can not be subdivided in progressive, persistent or regressive subtypes. Therefore most patients with CIN lesions are actually being treated as essentially premalignant, potentially progressive lesions. A technique that could distinguish between really potentially progressive and potentially regressive or reactive lesions would be very welcome for everyone involved.

In the Netherlands about 1200 new cases of cervical invasive squamous cell carcinoma occur per year (33,36). The prevalence of CIN I, II, and III lesions in the Netherlands as detected by cytological screening is not known precisely. It has been estimated that per year, in about 1% of women, in the age category 35 to 55 years, CIN I, II or III lesions may be detected (34). On a total population of 2,000,000 Dutch women in this age group, this signifies 20,000 cases of cytologically detected CIN lesions per year. Recently it was proposed by the Dutch Scientific Societies of Pathology and Obstetrics and Gynecology, that all cases of CIN II or III (and CIN I after one repeated positive smear), should be colposcopically evaluated (96). This would mean a very high number of colposcopical examinations and as a likely consequence a large increase in the number of histological examinations, conisations, laser evaporizations, cryocoagulations, diathermic ablatios and hysterectomies a year. These figures indicate the importance of the development of effective progression markers in cervical cytology. If the statement of Koss is right that only 10% or less of all CIN lesions will progress into invasive carcinoma, such progression markers could give a large saving on the emotional and financial costs. The subject of this thesis is the assessment of the possible role of DNA-cytometry for the detection of progressive and non-progressive cervical intraepithelial lesions. The possible role of human papilloma virus will be discussed briefly.

HUMAN PAPILLOMA VIRUS

Epidemiologic studies have shown that cervical carcinoma behaves as a venereally-transmitted infection in which one or more agents are transmitted to the women by male patients (51,54,86). High risk factors for cervical carcinoma are low age at the onset of sexual activity and having had multiple sexual partners or sexual

promiscuity. Coppleson and Reid suggested that spermatozoa may trigger neoplastic events in the cervical epithelium (22). Transmissible agents that have been studied are treponema pallidum, diplococcus gonorrhoea, trichomonas vaginalis, herpes simplex virus type 2 (HSV 2) and human papilloma virus (HPV).

The human papilloma virus is closely related to the SV40 virus and Shope papillomavirus, which can produce malignant tumors in animals. It is thought that HPV has a potential as an oncogenic virus in the human. No tissue culture system exists however that could support the growth and maturation of HPV in vitro. The relation between HPV infection and CIN and cancer has been based purely on observations made in tissues in vivo (1,25). The presence of HPV may be suggested in cervical smears by koilocytes, squamous cells with enlarged hyperchromatic nuclei and large clear perinuclear zones which are sharply demarcated against the peripheral cytoplasm (55,65,81). Other features that can be seen in epithelial lesions related to HPV, are multinucleation and individual keratinization, parakeratosis, epithelial pearl formation, acanthosis, frequent mitotic figures and prominent rete pigs (53,67). These histopathological features, that have been described as flat condyloma or subclinical papillomavirus infection (SPI) may be seen intermingled with changes present in CIN lesions or adjacent to CIN lesions (66,83). The virus can be demonstrated in biopsies and cervical smears by electronmicroscopical detection of HPV specific spherical crystalline particles with a diameter of 52 to 55 nm (43,61) or by immunochemical techniques, demonstrating viral capsid proteins mostly in the intermediate and superficial epithelial layers (48,60,99). Using molecular hybridization techniques such as Southern blotting, blot hybridization, and in situ hybridization, HPV DNA has been demonstrated in cervical intraepithelial neoplasia and invasive carcinoma. Approximately 50 types of HPV have been identified to date (18,25,30,63,72,79,101,102). Reviewing the literature, Zur Hausen concluded that HPV type 16 is found in approximately 50% of all cervical, penile and vulvar cancers, and HPV 18 in close to 20% of these lesions (102). It has been suggested that HPV may play a role as a promotor. Neoplastic transformation might be determined by specific HPV types but, it requires an initiation by other carcinogenic stimulus, e.g. herpes simplex virus type 2 and/or cigarette smoking (18).

The recognition that human papilloma virus infection is associated with cervical precancerous lesions and invasive carcinoma has raised the hope that identification of the virus may have prognostic value (23,56). Boon and Fox compared the natural course of CIN lesions in 100 women with and without koilocytosis No

differences of behavior could be demonstrated during 3-4 years follow-up (17). Meisels et al described that nearly 70% of lesions suggestive of HPV infection disappeared after a follow-up period of 15 months (68). The significance of HPV infection as a progression marker has to be further evaluated by prospective trials. Preliminary findings of the HPV studies have not led to a change in attitude towards CIN lesions. Bonfiglio and Stoler in an editorial in Human Pathology recently stated, that "these lesions should be classified and reported to the degree of morphological abnormality present, irrespective of the presence or absence of histological or other evidence of HPV infection" (16). Clinical evaluation and mode of treatment should be chosen irrespective of evidence of the absence or presence of HPV or a specific genotype (16,18,22,56).

DNA ANALYSIS

For many years it is known that malignant tumors frequently have chromosomal changes. This has recently attracted much attention as a result of three major developments. 1) In solid tumors, chromosomal rearrangements, usually translocations are uncovered, which are based on exchange of material between two, or occasionally more, chromosomes following breakage (40). Some of the translocations appear to be specific and may play a role in the initiation of the neoplastic process, as indicated for chromosomes #8 and #14 in the study of Burkitt lymphoma (44,100). Genic changes may be mediated by numeric or structural chromosomal changes. Numeric changes indicate the gain or loss of whole chromosomes. Structural changes are various and include translocations between chromosomes and other forms of rearrangement of chromatin. As a result of these changes portions of chromosomes may be present in excess or may be deficient. The discovery of a shortened chromosome #21 in chronic myelogenous leukemia was of special of interest, because it often occurred as the only anomaly in a predominantly diploid malignancy. Because of this phenomenon this #21 deletion was regarded as strong evidence to link genetic alteration and the etiology of cancer. 2) It is suggested that activation of particular genes (oncogenes) is important in neoplastic transformation. The function of genes located in the vicinity of breakage-points may be modified. Some oncogenes are situated close to breakage-points of chromosomes that take part in translocations, as in Burkitt lymphoma (44,100). It is believed that the activation of these oncogenes is a direct

consequence of their transference to a new chromosomal site following translocation. The activation of oncogenes may be brought about in various ways, e.g. by their interaction with viruses (3,44). The increased interest for this type of DNA analysis has led to the discovery of many oncogenes and their possible role in uncontrolled cell proliferation. 3) The recent descriptions of tumor suppressor genes have indicated that loss of genetic functions may furthermore be critical for the development of cancer. A finding that was supported by the results of studies of restriction fragment length polymorphism's (RFLP's), that showed that specific chromosomal deletions are often associated with certain malignancies (62,87,92).
It has to be stated however that cytogenetic studies of solid tumors, such as cervical invasive squamous cell carcinoma and cervical intraepithelial neoplasia, still are very complicated and time consuming, and cannot be used in a clinical setting. In solid tumors metaphases are relatively infrequent and it is difficult or even impossible to obtain good chromosome spreads and satisfactory chromosome banding. Furthermore chromosome changes in solid tumors are often very complex. In situ hybridization, a technique, that is developing very rapidly, does allow the study of complete chromosomes and details of chromosomes in interphase nuclei in frozen sections, and in paraffin-embedded sections. This technique has the potential to become clinically applicable (24,45).
Other techniques to study DNA changes which may become applicable in a clinical setting, are DNA ploidy analysis and high resolution DNA cytophotometry, based on measurements of cells stained with DNA-specific stains, such as the Thionin-Feulgen stain (73).

DNA ploidy analysis.

It is generally accepted that a developing tumor has a dominant stemline with a particular karyotype, within which variants are continually being produced. Many of these variants will not survive while others may remain viable and are directly related to more malignant properties of the tumor. This proces continues during lifetime of the tumor and may give rise to subclones, which predominate in certain regions of the tumor or may even take over as a new stemline. It is possible for the entire (or for part of the) chromosome complement to be doubled in some regions of the tumor. The result of this polyploidization may be the emergence of a stemline with a high basic chromosome number in the triploid or tetraploid region. As the proces of polyploidization continues a subpopulation of cells with

the double number or higher multiples of the basic chromosome number may develop. It is suggested that a tumour cell line with double the original chromosome set becomes dominant and that loss of chromosomes then produces the hypotetraploid values which are thought to be common in cancer (3). DNA ploidy analysis can be performed by flow cytometric and image cytometric (cytophotometric) techniques. Flow cytometry allows DNA ploidy analysis of a large number of cells in a short time, but it is not possible to study chromatin texture or geometric features of individual cells in detail. Image cytometric analysis also allows DNA ploidy analysis, but may also give information of the chromatin pattern and geometry of the nuclei. The number of cells that can be measured are limited and the speed of measurements is still lower.

The flow or image cytometric DNA ploidy patterns can be visualized by histograms displaying the amount of DNA present in the stemlines (42). The histograms may be assessed as DNA-diploid, DNA-polyploid and DNA-aneuploid patterns, according to the following definitions (7,41,42). In a diploid(like) pattern a distinct G0/G1 peak (corresponding to cells in rest phase of the cell cycle) is found in the (near-)diploid (2C; $0.9<DI<1.1$) region with a small proportion of cells in S mode (corresponding to the synthesis phase) and in G2/M mode (corresponding to the G2 phase and mitotic phase), a pattern which is very similar to that of normal tissue. In polyploidy distinct peaks are present in the diploid and tetraploid (4C; $1.8<DI<2.2$) regions, or in the diploid, tetraploid and octaploid (8C; $3.6<DI<4.4$) regions. Very few cells or none at all have DNA values corresponding to the DNA synthesis phase of normal cells. In all other cases ploidy patterns are considered aneuploid.

An additional description of the DNA content of a population of cells may be obtained by the calculation of the 2.5c and 5c exceeding rates. These are defined as the percentages of cell nuclei having DNA values higher than 2.5c (1.25 x normal diploid amount of DNA) respectively higher than 5c (2.5 x normal diploid amount of DNA) (15,71).

DNA ploidy patterns in normal tissues.

The normal tissues of the body are composed of cells with multiples of the haploid chromosome set. The gametes are haploid, most other cells are diploid, a few are tetraploid and other multiples going in powers of two (like megakaryocytes). It is assumed that loss of chromosomes, that may be encountered sometimes in normal tissue can be explained by loss during the technical preparation

of the tissue (3,91). In bone marrow however chromosome loss (e.g. 45 instead of 46 chromosomes) is seen with advancing age. Spriggs stated that apart from chromosomal loss and the presence in the same tissue of polyploid cells, there is no reason to suspect that deviations from the normal karyotype commonly occur (91).

DNA ploidy patterns in cervical invasive carcinoma.

As already stated, a complete karyotypic analysis of cervical tumors is frequently impossible because of technical difficulties. Nevertheless it was shown that numerical changes occur in cervical cancer, and although being extremely variable, they appeared to be non-randomly distributed (2,90). Thus it could be determined that certain chromosomes were especially involved in structural or numerical changes: Chromosome #1: excess of part of the long arm or of the whole chromosome in a near-diploid tumor; Chromosomes #3, #6, #17: often structural changes, Chromosome #11: structural changes or deletion of chromosome. Several tumors had small abnormal metacentric marker-chromosomes of uncertain origin. (3,5,6, 49,94). Jones, using the direct squash technique on cervical biopsies of four cases of squamous cell carcinoma, reported that the modal number was in the diploid (47) range in one case and in the near-triploid (65-67 chromosomes) range in the other three cases (49). Vang Nielsen indicated that errors in chromosome counting procedures are likely to occur and that cytometric DNA ploidy analysis may overcome this problem by measuring the relative DNA content of all cells in the specimen (94). Most ploidy analysis studies were performed by cytometric techniques. In a cytometric DNA ploidy analysis study of 468 cases of cervical invasive squamous cell carcinomas, Atkin demonstrated that just over half the cases fell into a near-diploid group and just under half into a triploid-tetraploid group (3). Of patients with near-diploid tumors 39% survived 5 years, of those with near-triploid and -tetraploid tumors 48%. Atkin raised the possibility that tumors with high modal values might be more radiosensitive (3,4). Jakobsen described the flowcytometric analysis of 171 cases of cervical invasive carcinoma, indicating that the rate of recurrence was highest in tumors with a DNA index >1.5 and that survival was highest with a DNA index <1.5 (47). Also Goppinger and coworkers could not confirm Atkin results. In a cytomorphometric study of cervical smears of 30 patients with squamous cell carcinoma, four patients with diploid and 6 with polyploid tumors were all alive after 10 years follow-up. Of the 20 cases with an aneuploid DNA pattern 14 had died and three were alive after 10 years follow-

up (32). Cervical carcinomas in the study by Atkin, tended to be more often near-diploid in younger patients, and more often near-triploid and -tetraploid in older patients (3). Also Strang and coworkers found age related differences in patients with squamous cell carcinomas; in patients of 35.6 (+/- 11.7) years of age tumors mostly had a diploid or polyploid DNA pattern and in patients 73 (+/- 12.6) years of age tumors were grossly aneuploid (93).

DNA ploidy patterns in cervical intraepithelial neoplasia.

Few chromosome studies have been performed in CIN lesions, mostly on un-banded preparations. In a study of 58 lesions Aktin demonstrated the presence of chromosome abnormalities and of cell clones with DNA values in the near-triploid or near-tetraploid region, as well as in the near-diploid area (3). Jones and coworkers reported the results of six cases of CIN using the direct squash technique. Three of these had a diploid (46-47 chromosomes) modal number of chromosomes, and in the other three cases a near-tetraploid (86-94 chromosomes) pattern was present (49). Spriggs and coworkers studied 28 cases of CIN and microinvasive carcinoma, and concluded that no chromosomal feature was distinctive of dysplasia, carcinoma in situ, microinvasive or invasive carcinoma (89). Spriggs and Atkin, both mentioned that marker chromosomes appeared to be infrequent, but stated that these are possibly more frequent in lesions that have progressed to the microinvasive stage (3,89). In a study in which banding techniques had been used, abnormal chromosomes were seen in two cases of carcinoma in situ (3). In both lesions abnormal chromosomes #1 were observed. In a case of microinvasive carcinoma an isochromosome #17 was noted. Atkin mentioned furthermore that carcinomas in situ may have undergone less chromosomal loss and fewer structural changes than invasive cervical carcinomas.

Microspectrophotometric, flow and image cytometric DNA ploidy measurements have been performed in cervical scrapings, fresh biopsy material and paraffin sections (3,12,13,14,28,29,37,46,71,69,85,98). Jakobsen and coworkers performed flow cytometric analysis of cervical biopsies and found that a DNA-aneuploid pattern was present in 7% of CIN I and II lesions and in 79% of CIN III lesions (46). Wilbanks and coworkers in an earlier report using two-wavelength Feulgen cytophotometry of cervical biopsies, mentioned that the degree of deviation from the DNA-diploid pattern of normal epithelium was least in mild dysplasia and became more pronounced in the higher grade lesions (98). In a (microspectrophotometric) study of cervical smears consistent with moderate dysplasia Nasiell and

coworkers could however not find significant differences between those cases that progressed to carcinoma in situ and those that regressed to normality (67). In a more recent paper Nasiell et al reported on a group of nine patients with cervical smears representing mild or moderate dysplasia, and showed that progression to carcinoma in situ was accompanied by a sequence of increasingly abnormal DNA patterns. In three patients with cervicitis as the final diagnosis, DNA patterns were within normal limits (71). Some investigators have implied that cervical intraepithelial lesions with an aneuploid DNA pattern have higher rates of persistence or recurrence than lesions with a diploid or polyploid pattern (13,29). The results of these studies seem to be in conflict with the DNA analyses showing (peri)-diploid or a low ploidy DNA patterns in more than 50% of micro- and macro-invasive squamous cell carcinomas (4,47). Invasive carcinomas with a low ploidy pattern were further reported to have a higher frequency of lymph node metastases than occurred in high-ploidy carcinomas of comparable size and depth of invasion (2,4,47). Therefore, more studies would have to be carried out before a new classification of Cervical Intraepithelial Neoplasias could be based on DNA ploidy pattern analysis.

High resolution cytophotometric analysis.

The cytological and histological diagnosis of malignancy is mainly based on changes in nuclear characteristics, such as differences in nuclear size, shape and chromatin distribution. The chromatin consists of DNA that forms a complex with proteins, mainly histone and non-histone proteins. The distribution of nuclear chromatin reflects the distribution of chromosomes, and as such the distribution of DNA throughout the nucleus (24). Cytologically in tumor cells an increase of heterochromatin, that reflects the compact chromatin, can be observed as a coarsening of the chromatin pattern.

Using a scanning microscope the chromatin texture, density and geometry of nuclei can be measured. Nuclei, processed with a specific Feulgen DNA stain, can be raster scanned by a finely focused light spot and stored as arrays of numbers in a computer system. Each number expresses the amount of light absorbed at the corresponding picture element or "pixel", and thus is a measure of the local DNA content. Differences in grey level between object and background be studied. Boundaries of the nuclei can be automatically determined from the digital image by grey level boundary following. Nuclear features, describing geometrical, density

and texture characteristics may be extracted from the digitized image (8,9,30,39,74,-80) Cytophotometric analysis has been of value in the study of cervical intermediate cells as present in cytological smears containing normal, dysplastic and carcinoma in situ cells, in studies of normal and malignant endometrium, in the differential diagnosis in pleural effusions and in the discrimination between classic and variant type small cell lung cancer cell lines (11,19,50,73,74,97). An advantage of DNA-cytophotometric analysis is that interphase tumor cells can be measured, providing objective information of chromatin distribution, which is an expression of chromosomal changes. Furthermore an implicit advantage of the high resolution cytophotometrical analysis is that large samples of digitized nuclei can be compared by sophisticated statistical techniques.

OUTLINE OF THE STUDY

The aim of the study was to investigate the potential of combined DNA ploidy analysis and high resolution cytophotometric analysis to discriminate between progressive and non-progressive cervical intraepithelial neoplastic lesions. Chapter 2 describes a technique to perform flow and image cytometric analysis on selected areas in paraffin-embedded tissues. This technique creates the possibility to study archival material in detail. Chapter 3 reports the application of flow and image cytometric DNA ploidy analysis, using this technique, on a group of paraffin-embedded CIN III lesions, in part with and in part without synchronous cervical invasive carcinoma. In chapter 4 DNA ploidy analysis and high resolution cytophotometric image analysis are performed in a group of CIN III lesions and a group of cervical invasive squamous cell carcinomas. Chapter 5 describes the application of high resolution cytophotometric analysis in CIN III lesions with and without synchronous cervical invasive carcinoma, in an effort to make a subdivision in potentially progressive and non-progressive CIN lesions. Chapter 6 compares the results of DNA ploidy analysis, 2.5c and 5c exceeding rates and high resolution cytophotometric analysis in CIN III lesions from cytological smears and in cytospin specimens, retrieved from selected areas in paraffin-embedded tissues. Chapter 7 reports the application of DNA ploidy analysis, 2 5c and 5c exceeding rates and high resolution cytophotometric analysis in cytological smears, representing a group of progressive CIN I-III lesions and a group of CIN III lesions with unknown outcome.

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CHAPTER 2

EXTRACTION OF NUCLEI FROM SELECTED REGIONS IN PARAFFIN-EMBEDDED TISSUE

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Abstract A method for the extraction of nuclei from selected regions in paraffin-embedded tissue is described. Fifty-micrometer sections are cut, dewaxed, and rehydrated. For the final handling, the sections are manually transferred from one tray to another. The sections are put on a slide under a dissection microscope and the region of interest is isolated by scrapping off the irrelevant region with a scalpel. An optimal number of single nuclei is obtained by incubation in a protease solution with intermediate syringing. The nuclei are washed and can be used for flow cytometry. Resuspension of the nuclei in foetal calf serum and cytocentrifugation results in preparations suited for image analysis. DNA cytometric measurements of nuclei in a carcinoma in situ and an invasive carcinoma region in breast tissue present in the same tissue block and in a severe dysplasia/carcinoma in situ (CIN III) region of the cervix are presented.

INTRODUCTION

The method for extracting nuclei from archival paraffin-embedded tissue (1,2,7-9) has made retrospective studies on large series of patient material possible. By the use of this method, nuclei are isolated from thick (30-100 μm) sections and used both for flow (1,2,7,8) and image (9) cytometry. With this method the correlation between the DNA content of tumor cells and the prognosis (3,4) for a number of malignancies has been determined. In addition to the use of this method in retrospective studies, it has other advantages. The cell composition of the thick tissue section is known beforehand by visual inspection of haematoxylin-eosin (H-E)-stained 4-7 μm sections cut before and after the thick section(s). This

check for cell composition avoids sampling errors and can also be used to select regions of interest. For instance, stromal and necrotic tissue in tumors, interfering with the measurements, can be eliminated in advance. Furthermore, nuclei from morphologically different regions in the tissue can be extracted.
This study explores the latter possibility. A method for isolating nuclei from well-defined regions in paraffin-embedded tissue is described. The method has been applied to breast and cervical tissue. Quantitative DNA measurements in nuclei obtained from these regions are shown as an example.

MATERIALS AND METHODS

Paraffin-embedded tissue was cut according to the following sequence: 5 μm, 2 x 50 μm, 5 μm, 2 x 50 μm, etc. The number of sequences was determined by the number of thick sections desired. The 5 μm sections were H-E stained and served as a control for cell composition. During the cutting of the thick sections, care was taken that they remained stretched, since once rolled up it was difficult to stretch them again. The sections were put into a small, round, stainless sieve (3 cm diameter, 1 cm high) with a nylon mesh with 100-μm holes in the bottom. They were dewaxed and rehydrated according to the following sequence: xylene (twice), 100% ethanol (twice), 80% ethanol, 70% ethanol, 50% ethanol, and distilled water. The sieves with the sections were put in petri dishes and kept for at least 10 min in each solution. Thereafter the sections were put on a slide. With the aid of the H-E-stained 5 μm sections, cut before and after the 50 μm sections, the cell composition and architecture in the thick sections were known. Together with this information as well as the microscopically observed tissue-cell architecture in the unstained thick section the area of interest was identified. Under a dissection microscope the irrelevant areas was scraped off with a scalpel and discarded. The tissue of interest was then scraped off and put into a centrifuge tube. The sections were incubated in phosphate-buffered saline (PBS; 137 mM NaCl, 13 mM Na_2HPO_4-$2H_2O$, 3 mM KH_2PO_4, pH = 7.4) with protease (Type VII from Bacillus amylolique faciens, Sigma Chemical Co., St Louis, MO) at 37°C. For breast tissue a 0.05% protease concentration and a 30-min incubation time were used. For cervical tissue a 0.1% protease concentration and a 60-min incubation time were used.
During incubation the sections were gently syringed at 15, 30, 45 and 60 min

with needles ranging in diameter from 1.1 mm to 0.4 mm. Incubation was terminated by adding 4-5 ml cold (4°C) PBS, after which the tubes were put in ice. The nuclei were washed twice with PBS with intermediate centrifugation steps. The nuclei were counted with a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, FL). For cervical tissue about 10,000 nuclei were centrifuged, the supernatant was discarded, and 150 μl foetal calf serum (normal pooled; Gibco, Paisly, U.K.) was added. The pellet was resuspended and placed on a slide with a cytocentrifuge apparatus (Shandon, U.K.; 10 min x 500 rpm). The slides were briefly (10-20 s) air-dried and fixed in a methanol 37% formaldehyde-acetic acid mixture (85:10:5 by volume) for 1 h. They were then Thionin-Feulgen stained and measured for DNA content (200 nuclei/slide) as described elsewhere (6).
For breast tissue enough nuclei were taken to prepare a slide for Thionin-Feulgen staining and measurement (150 nuclei/slide). The remainder of the nuclei was centrifuged and fixed in cold (-20°C) 70% ethanol. The nuclei were then stained for DNA with propidium iodide (5) and measured in a cytofluorograph system 50H flow cytometer (Ortho Instruments,Westwood,MA).

RESULTS

Figure 1 shows an example of nuclei of a severe dysplasia/carcinoma in situ (CIN III) region of the cervix, prepared according to the standard procedure. As can be seen the chromatin texture is well preserved.
Figure 2A shows a picture at low magnification of a 5-μm section of a breast tumor. The upper portion was diagnosed as an invasive lobular breast carcinoma (see Fig. 2B) and the lower part was diagnosed as lobular carcinoma in situ (CIS) (Fig. 2C). Figure 3a shows a DNA histogram obtained by flow cytometry and Figure 3d shows a DNA histogram obtained by image (scanning) cytometry of nuclei from the entire (nonselected) thick tissue section cut after the 5-μm section shown in Figure 2A. As can be clearly seen in both figures, aneuploidy is present. Figure 3b shows a flow DNA histogram and Figure 3e shows an image DNA histogram of the cells from the CIS region. In this case diploidy is found. DNA histograms of the invasive carcinoma region are shown in Figure 3c (flow) and 3f (image). Here again, as in Figure 3a, aneuploidy is observed.

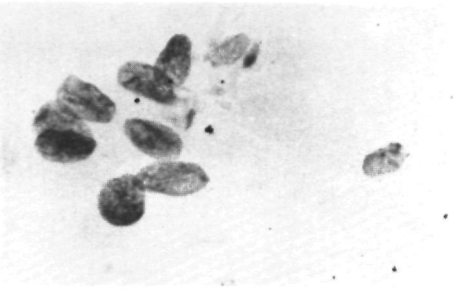

Figure 1: Isolated nuclei from a severe dysplasia/carcinoma in situ (CIN III) region of the cervix, prepared according to the standard procedure (Thionin-Feulgen stain, x660).

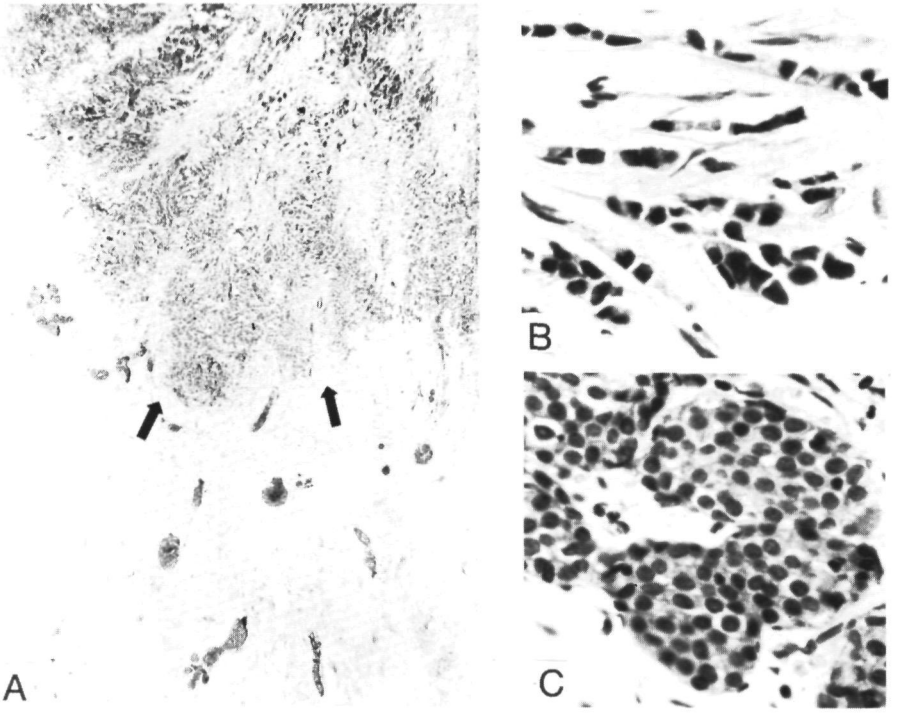

Figure 2 A: Microscopic picture at low (x21) magnification of a lobular breast carcinoma with an invasive carcinoma (top, above the arrows) and a CIS (bottom) region.

B: Picture at high (x430) magnification of the cells in the invasive carcinoma region of A.

C: Picture at high (x430) magnification of the cells in the CIS region of A.

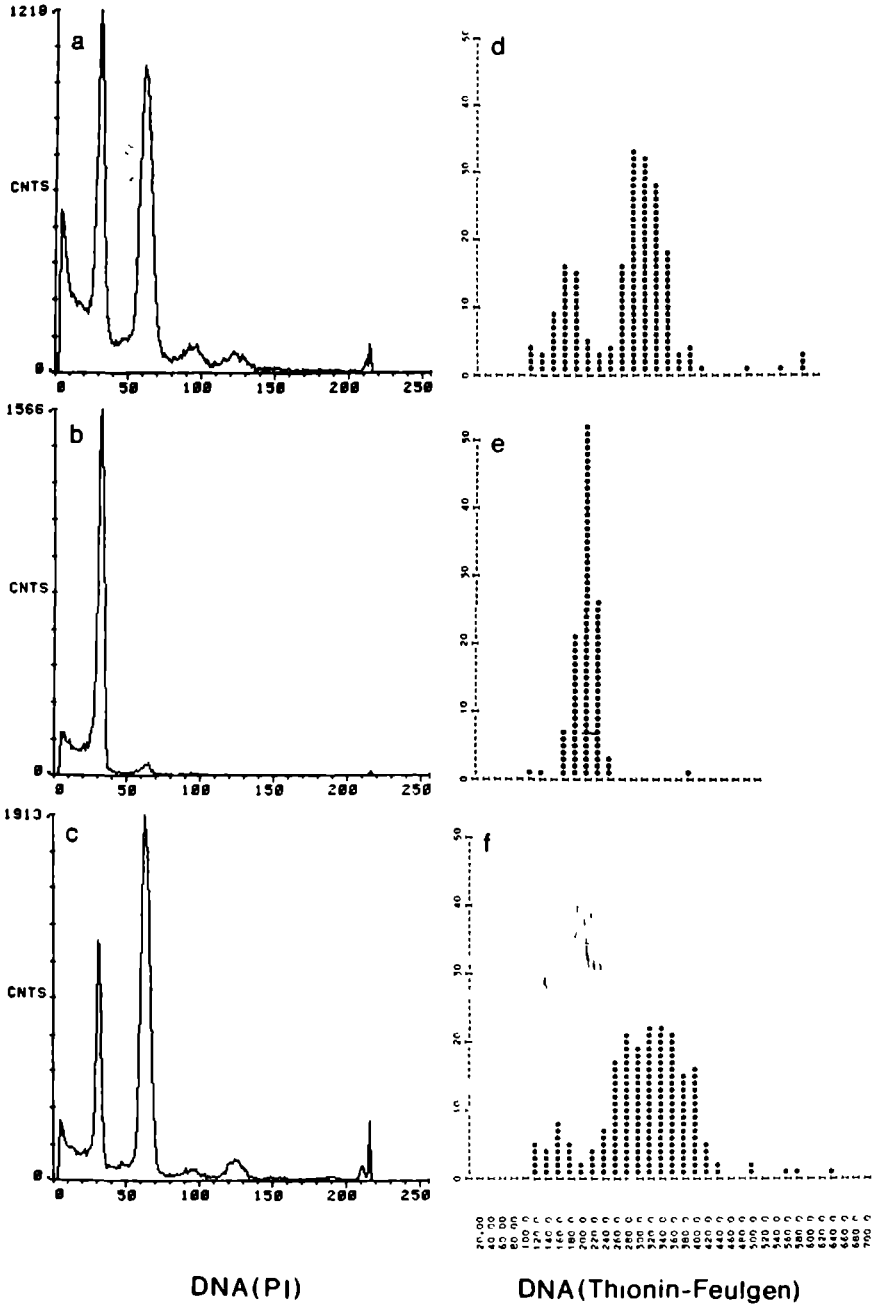

Figure 3: Propidium iodide DNA flow cytometry (a-c) and Thionin-Feulgen DNA image (scanning) cytometry (d-f) of the breast carcinoma in Figure 2. a and d are histograms of the entire section, b and e are from the CIS region, and c and f are from the invasive carcinoma part.

Figure 4A shows a picture at low magnification of cervical tissue with CIN III on the surface (between the arrows) and stromal tissue underneath, composing more than half of the total tissue block. Figure 4B shows the cells of the CIN III region at higher magnification.

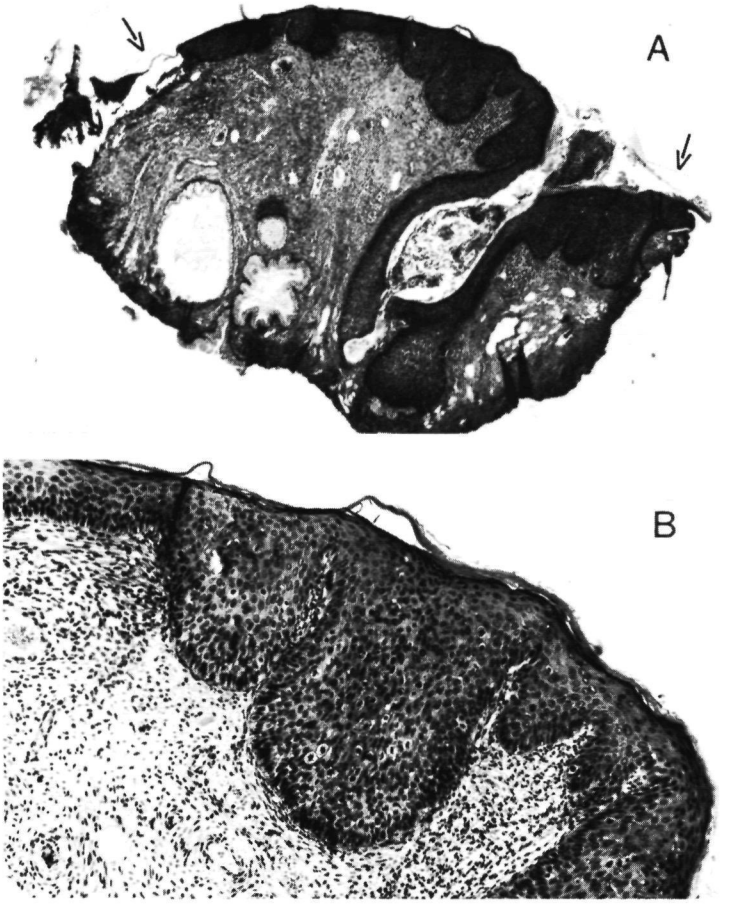

Figure 4: Microscopic picture at low (x15) magnification of cervical tissue with a CIN III region (between the arrows, at the surface). B. Picture at high (x76) magnification of the CIN III cells in A.

Figure 5a shows an image (scanning) cytometric-derived DNA histogram of nuclei from the entire thick section. As can be seen, diploidy is observed with some aneuploid cells. Figure 5b shows a DNA histogram of the nuclei obtained from the CIN III region. A peak at 140 arbitrary units (A.U.) and a second at 280 A.U. can be observed. The nuclei were measured at random, thus including nuclei

of leukocytes and some stromal cells (fibroblasts). Since in scanning cytometry the nuclei can be visually inspected before measurement, clearly non-CIN III cells, such as leukocytes, can be omitted. Therefore, a third histogram (Fig. 5c) was constructed, in this case with the omission of leukocytes. A contamination in this histogram with some stromal cells is still possible. It was not possible to formulate morpholocial criteria to distinguish these from some oval- and longshaped CIN III cells.

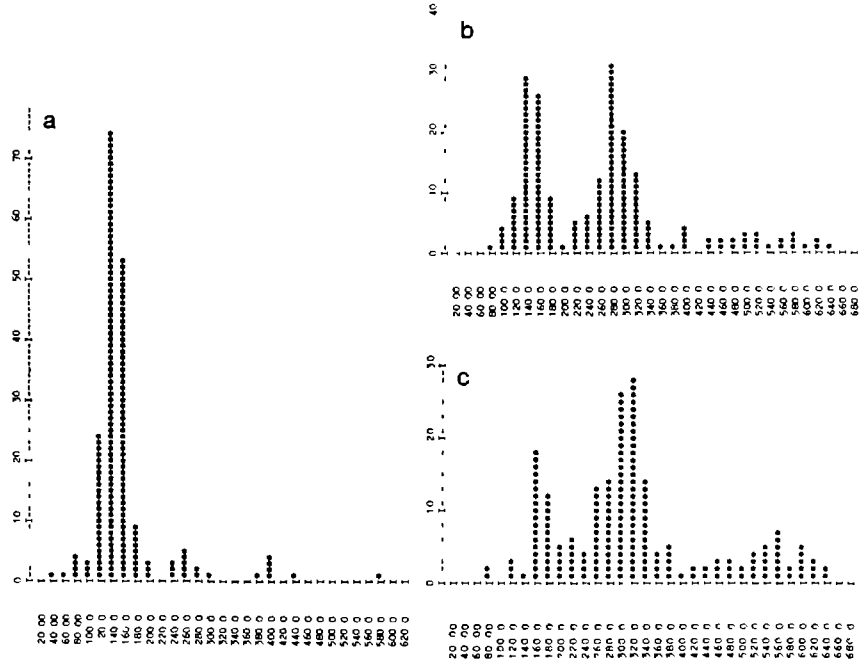

Figure 5 A·Thionin-Feulgen DNA image (scanning) cytometric histogram of all nuclei of the entire section shown in Figure 4A. B DNA histogram of all nuclei in the CIN III region. C DNA histogram of all nuclei in the CIN III region, with the omission of leukocytes.

DISCUSSION

In this paper a nuclear preparation method is evaluated that prepares nuclei from (small) selected areas in paraffin-embedded tissue. After testing different enzymes, e.g., pepsin (1,2), trypsin (7), and protease (9), the latter was found

to be optimal. Pepsin and trypsin could be used when only flow cytometric DNA measurements were performed. However, when nuclei had to be put on slides for image analysis measurements, it appeared that the chromatin texture was disrupted by these enzymes. Protease resulted in nuclei of optimal morphology (Fig. 1). These results agree with those found by Van Driel-Kulker et al. (9). Flow cytometric DNA measurements after protease treatment were comparable to those found with pepsin or trypsin (results not shown).

The concentration and incubation time for the protease treatment were different, depending on the tissue type. For cervical tissue both concentration and incubation time had to be doubled compared to that for breast tissue. A concomitant syringing procedure was necessary for an optimal number of single nuclei for both tissues. These results differ from those reported by Van Driel-Kulker et al. (9). They reported that a fixed concentration and incubation time without syringing was sufficient for an optimal number of nuclei for the different tissue types they tested.

As can be concluded from the results, more information is available when measurements in nuclei from selected regions are performed than in nuclei from the whole thick tissue sections. In the examples of the breast tumor, the information that the CIS region is diploid (Fig. 3b,e) and the invasive carcinoma part aneuploid (Fig. 3c,f) is lost when nuclei from the whole section are measured (Fig. 3a,d). It should be stressed that in this example the CIS part is diploid and the invasive carcinoma part aneuploid; this does not have to be a general phenomenon.

In the example of the cervical tissue, not much information is present in the DNA histogram of the entire tissue (Fig. 5a); only some aneuploid cells are detected. When the nuclei from the CIN III region are measured randomly, a DNA histogram is obtained that is clearly interpretable (Fig. 5b). A first diploid peak is found at 140 A.U. while a second tetraploid peak is found at 280. The cells with higher DNA values are probable cells in S and G_2M phases of the tetraploid population. Most likely all CIN III cells are tetraploid as can be concluded from the histogram in Figure 5c. The small diploid peak at 140 A.U. is most probably from stromal cells that cannot be distinguished from CIN III cells.

The procedure as described in this paper is now in routine use in our laboratory. In most breast tumors, enough tissue is available to perform both flow and image cytometry. For the cervical tissue, too few nuclei are obtained from most CIS

regions to perform flow cytometry but are sufficient to perform image analysis. The method, as described in this paper, has been illustrated by quantitative DNA measurements on nuclei extracted from these regions. However, more types of measurements are possible: for instance, multiparameter flow cytometry as well as image analysis measuremets of the size, shape, and texture of the nuclei.

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CHAPTER 3

DNA PLOIDY PATTERNS IN CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III, WITH AND WITHOUT SYNCHRONOUS INVASIVE SQUAMOUS CELL CARCINOMA

Measurements in nuclei isolated from paraffin-embedded tissue

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Abstract This study presents the results of cytophotometric (CPM) and flow cytometric (FCM) DNA ploidy measurements in cervical intraepithelial neoplasias grade III (CIN III) with and without synchronous invasive squamous cell carcinoma. Hysterectomy and biopsy material from 21 patients 35 years of age or younger and from 18 patients age 50 years or older was studied. The DNA analysis was performed in nuclei isolated from specific areas of paraffin-embedded tissue. There were significant differences in the distribution of DNA patterns between the two age groups. About 80% of CIN III lesions in women 50 years of age or older, with or without a coexisting invasive cancer were aneuploid. In the group of younger women a diploid DNA pattern was found in about 60% of CIN III with concomitant invasive cancer. In the absence of an invasive cancer, CIN III lesions were mostly polyploid. The DNA pattern of invasive cancers was generally identical with the adjacent CIN, thus suggesting that the two lesions were related. Although the prognostic value of DNA ploidy measurements in cervical intraepithelial lesions in women in these two age groups has to be further evaluated, these results are at considerable variance with previously published data on DNA values in CIN and invasive carcinoma. In four CIN III lesions without invasive cancer, in women of the group of 35 years of age or younger, human papilloma virus common antigen could be demonstrated by immunochemical procedure. In three of these cases a polyploid DNA pattern was present; the fourth case showed a bimodal aneuploid pattern.

Epidemiologic studies of cervical neoplasia have shown the incidence of carcinoma in situ and related lesions, grouped as cervical intraepithelial neoplasia grade III (CIN III) to be much higher than the incidence of invasive squamous cell carcinoma (ISC), indicating that only a relatively small number of CIN III lesions will progress to invasion. The majority of CIN III lesions, therefore, will either regress or persist (1-3) Biopsies or treatment may account for some, but not all, of these cases (4-5).

It is not possible to separate CIN III lesions into regressive, persistent, or progressive subtypes purely on the basis of histologic, cytologic, or clinical criteria The use of objective parameters such as DNA measurements is being given increasing attention (6-13). Aneuploidy has been a frequent finding in CIN III lesions using chromsome analysis (6-7), cytophotometric (CPM) or flow cytometric (FCM) DNA measurements in cervical scrapings (8,9), fresh biopsy material (10), or paraffin sections (11,12)

Cervical intraepithelial lesions with an aneuploid DNA pattern have been reported to have higher rates of persistence or recurrence than lesions with a diploid or polyploid pattern (8,11,12). The results of these studies seem to be in conflict with DNA analyses which showed (peri-) diploid or low ploidy DNA patterns in more than 50% of micro and macroinvasive squamous cell carcinomas (13,14) Invasive carcinomas with low ploidy pattern were reported to have a higher frequency of lymph node metastases than occurred in high-ploidy carcinomas of comparable size and depth of invasion (6,8,13,15) Furthermore, it was stressed by Atkin (15) that a diploid DNA pattern does not necessarily indicate a favorable outcome.

Recently the role of human papilloma virus (HPV) infection in the pathogenesis of cervical neoplasia has attracted much attention. Along with koilocytotic atypia, which has been accepted as a pathognomonic cytologic marker for HPV infection (16-19), HPV DNA can be demonstrated in most cases of cervical intraepithelial neoplasia and ISC using molecular hybridization techniques (20-24). With immunohistochemical techniques, the presence of the common viral capsid antigen could be documented in some cases of cervical intraepithelial neoplasia, but not in invasive squamous carcinoma (24-26) The DNA ploidy pattern of the lesions associated with HPV was studied by Fu and co-workers (12,27). They stated that typical condylomas without a significant degree of nuclear pleomorphism have a

polyploid DNA distribution, whereas "those changes having an aneuploid DNA pattern are true neoplastic processes".

This article reports the results of DNA measurements performed on whole nuclei derived from specific areas of the uterine cervix identified under the microscope. The method used in this study allowed us to compare the DNA content in intraepithelial lesions classified as CIN III without adjacent invasive cancer (CIN) with the DNA content in CIN III-like lesions adjacent to invasive cancer (CIN-INV). Furthermore, we compared the DNA content in intraepithelial CIN III-like lesions adjacent to areas of invasive carcinoma with the DNA content of the invasive component. The study was performed on two groups of women: age 35 years or younger and age 50 or older, corresponding to the biphasic distribution of invasive carcinoma (2,3). In all patients representative histologic sections were stained for the presence of papilloma virus common antigen.

PATIENTS AND METHODS

Patients

In this study cervical tissue derived from hysterectomy and biopsy material of 39 patients was examined. The CIN III was defined as an epithelial lesion in which undifferentiated basal or parabasal cells with nuclear enlargement, crowding, indistinct cell boundaries, and a high nucleo-cytoplasmic ratio occupied more than two thirds (or the full thickness) of the epithelium (28). Superficial cytoplasmic keratinization could be present.

The lesions were divided into separate groups, according to age of the patients and the presence or absence of invasive cancer adjacent to intraepithelial neoplasia (Table 1).

The first group contained 11 cases of CIN III in patients 35 years or younger, without concomitant microinvasive or macroinvasive squamous cell carcinoma (I:CIN). The second group (IIA and IIB) consisted of ten patients also 35 years of age or younger, but with CIN III lesions (IIA:CIN.INV) and an invasive carcinoma (IIB:INV) directly related to the CIN lesions in the same or in an adjacent tissue block.

Table 1. Cervical epithelial abnormalities, subdivided for type of lesion and age.

	≤ 35 yr			≥ 50 yr		
	Pat. grp	Lsn. N	Pat. N	Pat. grp	Lsn N	Pat. N
Cervical intraepithelial neoplasia grade 3 without adjacent invasive carcinoma (CIN)	I	11	11	III	8	8
Cervical intraepithelial neoplasia grade 3 with adjacent invasive carcinoma (CIN.INV)	IIA	10	10	IVA	10	10
Invasive squamous cell carcinoma adjacent to CIN 3-like lesions (INV)	IIB	10		IVB	10	
Total		31	21		28	18

The CIN III lesions with or without concommitant invasive cancer were morphologically identical. However, the biological behavior is likely to be different. To differentiate between these two lesions we introduced for CIN III lesions with concomitant invasive cancer the term CIN III-like lesions (CIN.INV). Eight women 50 years or older with CIN III, without a concomitant invasive cancer constituted the third group (III:CIN).

The fourth group was composed of ten women, 50 years or older, with CIN III-like lesions (IVA:CIN.INV) and an invasive cancer in the same or in an adjacent tissue block (IVB:INV).

Using a recently described method for extracting intact nuclei from paraffin-embedded tissue (29-31), it is possible to perform image and FCM DNA measurements of preselected areas in tissue sections.

Thus using selected areas, DNA content of pure intraepithelial lesions (CIN) can be compared with DNA content of intraepithelial lesions directly adjacent to invasive squamous cell carcinoma (CIN.INV) (Fig. 1). Also, the DNA content of invasive carcinomas (INV) can be compared with DNA content of both types of CIN III (CIN and CIN.INV).

Method of extraction of nuclei from paraffin-embedded tissue

Two consecutive 50-μm sections were cut from paraffin-embedded formalin-fixed

tissue containing CIN III and/or invasive cancer. Fifty micrometers' thickness was proven to cause the least number of artifacts in DNA histograms (32). Sections were deparaffinized and rehydrated in the following sequence: xylene (twice), 100% ethanol (twice), 80% ethanol, 70% ethanol, 50% ethanol and distilled water (at least 10 minutes in each solution). Thereafter tissue sections were placed on microscopic slides. For orientation and localization of relevant areas 5-μm tissue sections were made before and after the two "thick" sections were cut. These were stained with hematoxylin and eosin (H&E). An example of a CIN III-like lesion with an adjacent invasive carcinoma is shown in Figure 1.

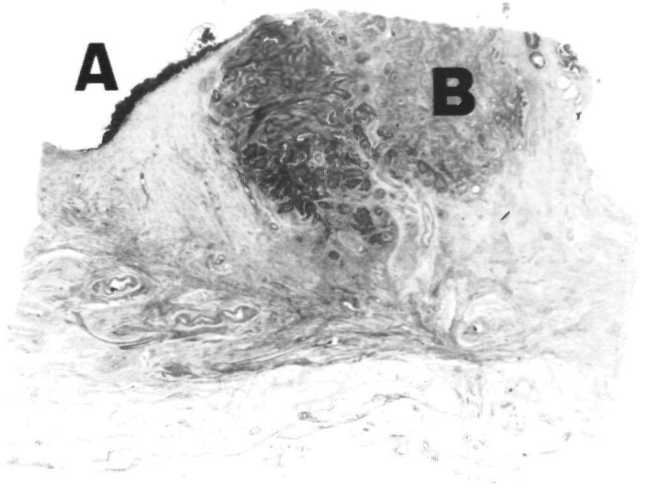

Figure 1: Invasive squamous carcinoma (B) and an adjacent CIN III (A) (H & E, x4).

Under a dissecting microscope the CIN III and/or invasive carcinoma regions were localized and prepared for DNA analysis by scrapping off nonrelevant areas with a scalpel. The target tissue was then taken from the glass slides, put in a centrifuge tube, and incubated in phosphate-buffered saline (PBS), with 0.1% protease (type VII from Bacillus amylolique faciens, Sigma Chemical Co, MI) at 37°C, for 60 minutes. During incubation the specimens were gently syringed using needles ranging in diameter from 0.4 to 1.1 mm (gauge, 23-19), at 15, 30, 45, and 60 minutes. Incubation was terminated by adding 4 to 5 ml of cold (4°C) PBS,

after which the tubes were put on ice. The nuclei were washed twice with PBS with intermediate centrifugation steps, and counted with a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, FL). About 10,000 nuclei were centrifuged, and 150 μl fetal calf serum (normal pooled Gibco, Paisly, UK) was added. This cell suspension then was centrifuged (5 minutes at 500 rpm), placed on slides using a cytocentrifuge (Shandon, UK) for 10 to 20 seconds, air dried, and fixed in a mixture of methanol, 37% formaldehyde, and acetic acid (85-10-5 by volume) for 1 hour.

Cytophotometric analysis

For CPM isolated nuclei from tissue blocks of all 39 patients were stained with Thionin-Feulgen (33). DNA content of 200 stained intact CIN III (CIN and CIN.INV) or invasive cancer (INV) nuclei was measured using the Nijmegen Image Analysis System (NIAS). The NIAS system is composed of an Orthoplan Scanning microscope (Leitz Wetzlar, West Germany), and provided with a linear diode array (Fairchild). The microscope is interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Maynard, MA). Nuclei were measured with monochromatic light with a wavelength of 585 nm (band width, 20 nm). Sampling density was four pixels per micrometer. As an internal standard for DNA content 20 lymphocytes per slide were measured.

Flow cytometric analysis

The suspension containing residual nuclei was centrifuged briefly and fixed in cold (-20°C) 70% ethanol.
The nuclei were then stained for DNA with propidium iodide (34) and measured in a Cytofluorograph 50H flow cytometer (Ortho-Instruments, Westwood, MA). In seven patients the number of cells was sufficiently high for both CPM and FCM analysis.

Histogram interpretation

The DNA content was expressed as DNA index (DI). DNA index was defined as the nuclear integrated optical density of the dominant peak or peaks of the histogram divided by the median integrated optical density of the "internal"

control sample of lymphocytes (35,36). In cytophotometry the DI distributions were defined from histograms using a bin size of 0.1. Histograms were classified as diploid, polyploid, or aneuploid (11,31). DNA ploidy patterns were designated diploid (2C;DI=1.0), when a distinct peak was found in the diploid or near diploid (DI-0.9-1.1) region, polyploid when distinct peaks were present either in the diploid (2C;DI=1.0), and tetraploid (4C;DI=2.0) or in the diploid, tetraploid and octaploid (8C;DI=4.0) regions. Ploidy patterns were considered to be aneuploid in all cases with scattered, unimodal, bimodal, or multimodal distribution of DNA peaks in the histograms.

Identification of papilloma virus infection

In all cases of CIN III and invasive cancer the presence of papilloma virus infection was studied. Paraffin sections (5-6 μm) were cut from the same tissue blocks and submitted for immunohistochemical staining with rabbit antisera, prepared against disrupted bovine papilloma virus type 1 virions (Dako Corporation, Santa Barbara, CA). These antisera react with the capsid antigens of both animal and human HPV (25-27). Immunohistochemical identification of papilloma virus common antigen was performed using the peroxidase-antiperoxidase (PAP) technique. Paraffin sections were deparaffinized, hydrated, treated with hydrogen peroxide in methanol, rinsed in PBS, preincubated for 10 to 30 minutes in normal swine serum, then incubated overnight with the antiserum, diluted 1:3000. The next day the specimens were treated with swine anti-rabbit (1:30) and PAP (1:100), and visualized with diamine benzidine (DAB). Slides with deep brown nuclei in two successive stainings were scored as positive for papilloma virus common antigen (25).

RESULTS

Comparison of flow cytometric with cytophotometric findings

In seven patients with invasive squamous carcinoma but in none of the CIN III cases, sufficient material was available for FCM DNA analysis. In all seven cases, results of FCM and CPM analyses were similar. Two examples are shown in figures 2A through 2D.

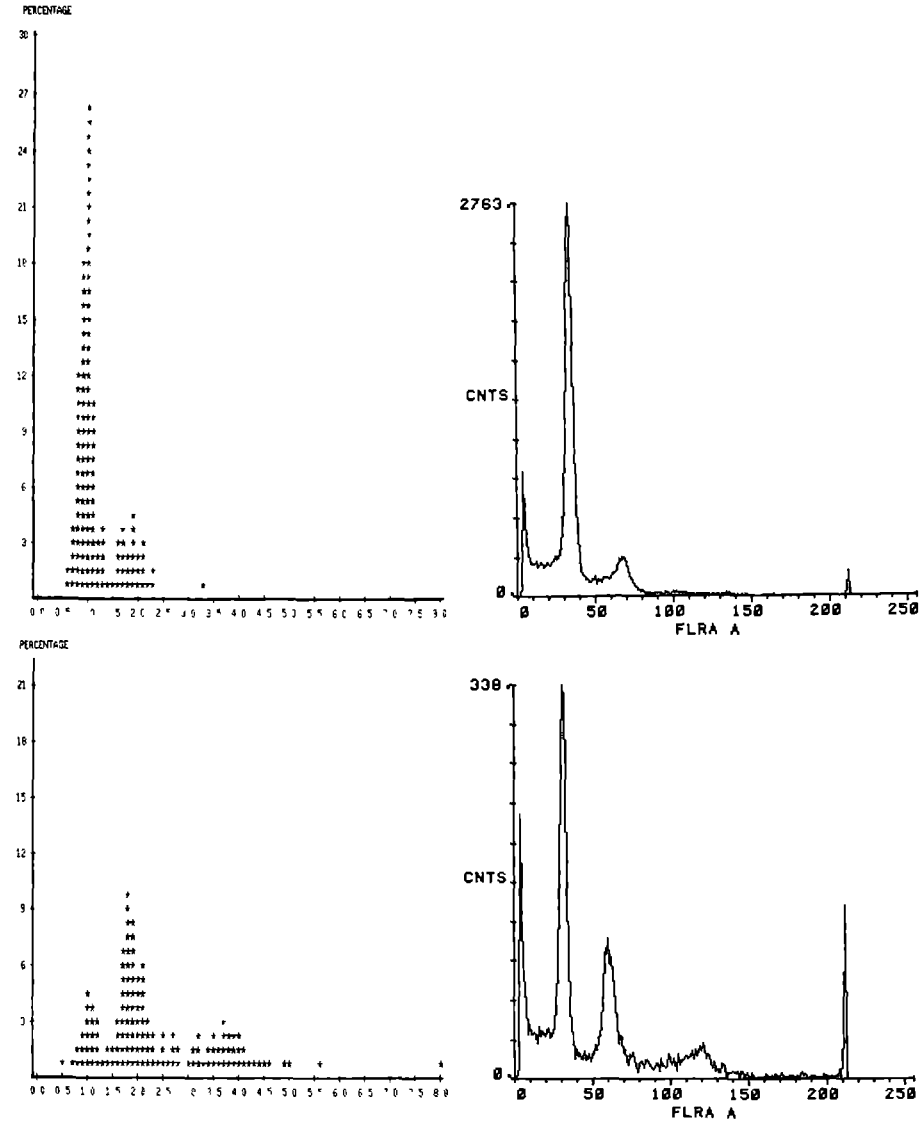

Figure 2A-2D: Diploid DNA pattern of an invasive squamous cell carcinoma is shown in both cytophotometry (CPM) (A, top left) and flow cytometry (FCM) (B, top right). The DNA index is shown on the X axis in cytophotometry histogram (woman 31 years of age). (C, bottom left) Cytophotometric (left) and (D, bottom right) flow cytometric histograms of an invasive cancer showing a polyploid DNA pattern. The first (diploid) peak is higher in the FCM histogram, because lymphocytes and stroma cells are measured as well (patient 31 years of age).

Cytophotometric results

Cytophotometric DNA analysis was done in all 39 CIN III lesions (CIN and CIN INV) and in 20 invasive cancers (INV).

The results of cytophotometric analysis of CIN III without adjacent invasive carcinoma (CIN), compared with CIN III with adjacent invasive carcinoma (CIN INV) are shown in Table 2.

*Table 2 DNA ploidy patterns in cervical intraepithelial neoplasia grade III with and without coexisting invasive squamous cell carcinoma**

	CIN			CIN INV		
DNA ploidy pattern	Total	≤ 35 yr	≥ 50 yr	Total	≤ 35 yr	≥ 50 yr
	N	N	N	N	N	N
Diploid	2 (11%)	2 (18%)	-	8 (40%)	6 (60%)	2 (20%)
Polyploid	7 (37%)	6 (55%)	1 (13%)	2 (10%)	1 (10%)	1 (10%)
Aneuploid	10 (53%)	3 (27%)	7 (88%)	10 (50%)	3 (30%)	7 (70%)
Total	19	11	8	20	10	10

*Total and subdivided for two age categories CIN cervical intraepithelial neoplasia grade III without adjacent invasive carcinoma, CIN INV CIN III with adjacent invasive carcinoma

In two cases of CIN the DNA histogram showed a diploid pattern, seven patients had a polyploid pattern, and ten an aneuploid pattern DNA analysis in 20 CIN III-like lesions with adjacent invasive carcinoma (CIN INV) disclosed a diploid DNA-pattern in eight cases, a polyploid pattern in two, and aneuploidy in ten cases.

When age of the patients was taken into consideration (Table 2), in CIN III lesions (CIN and CIN INV) an aneuploid DNA pattern was most frequently found in patients 50 years of age and older, both in the absence (seven of eight patients) and in the presence (seven of ten patients) of an adjacent invasive squamous cell carcinoma

In the younger age group (≤35 yr) aneuploidy was present in only three of 11 (27%) patients with CIN III and in three of ten (30%) patients with a CIN.INV lesion. A diploid pattern was observed in two (two of 11, 20%) cases of CIN lesions in younger patients. In patients with CIN INV lesions, diploidy was found

in six (60%) younger women and in two (20%) women older than 50 years. A polyploid DNA pattern was identified in six (six of 11, 55%) patients from the younger age group with a CIN lesion, only in one older patient (one of eight, 13%) with a CIN lesion and in patients with CIN INV lesions in one patient (10%) of each age group

Table 3 Distribution of DNA patterns in 20 cases of invasive cancer and adjacent cervical intraepithelial neoplasia grade III-like lesions

	CIN III-like lesion (CIN INV)			
Invasive cervical cancer (INV)	Diploid	Polyploid	Aneuploid	Total
Diploid	8	-	-	8
Polyploid		1	1	2
Aneuploid	-	1	9	10
Total	8	2	10	20

In Table 3 distribution of DNA patterns in 20 invasive squamous cell carcinomas (INV) and adjacent CIN lesions (CIN INV) is compared. In 18 of 20 cases (90%) DNA patterns in invasive carcinomas were similar to those of adjacent (CIN INV) lesions (eight diploid, one polyploid, nine aneuploid).

In figures 3A through 3D two characteristic examples are given. In the cases with DNA aneuploidy in both the intraepithelial lesion (CIN INV) and the adjacent invasive carcinoma, DNA distribution in invasive cancers usually was more scattered. In two cases (10%) DNA patterns of the intraepithelial lesion and the invasive cancer were different. In one case the (CIN INV) lesion was polyploid whereas the invasive cancer had an aneuploid pattern In the second case (Figs 4A and 4B) the opposite was noted

Identification of papilloma virus infection

Papillomavirus common antigen could be detected in four women younger than 35 years of age with a (CIN) lesion. Papillomavirus was not detected in any of the other lesions.The DNA pattern was polyploid in three of these four younger patients In the fourth patient a bimodal aneuploid DNA pattern was seen

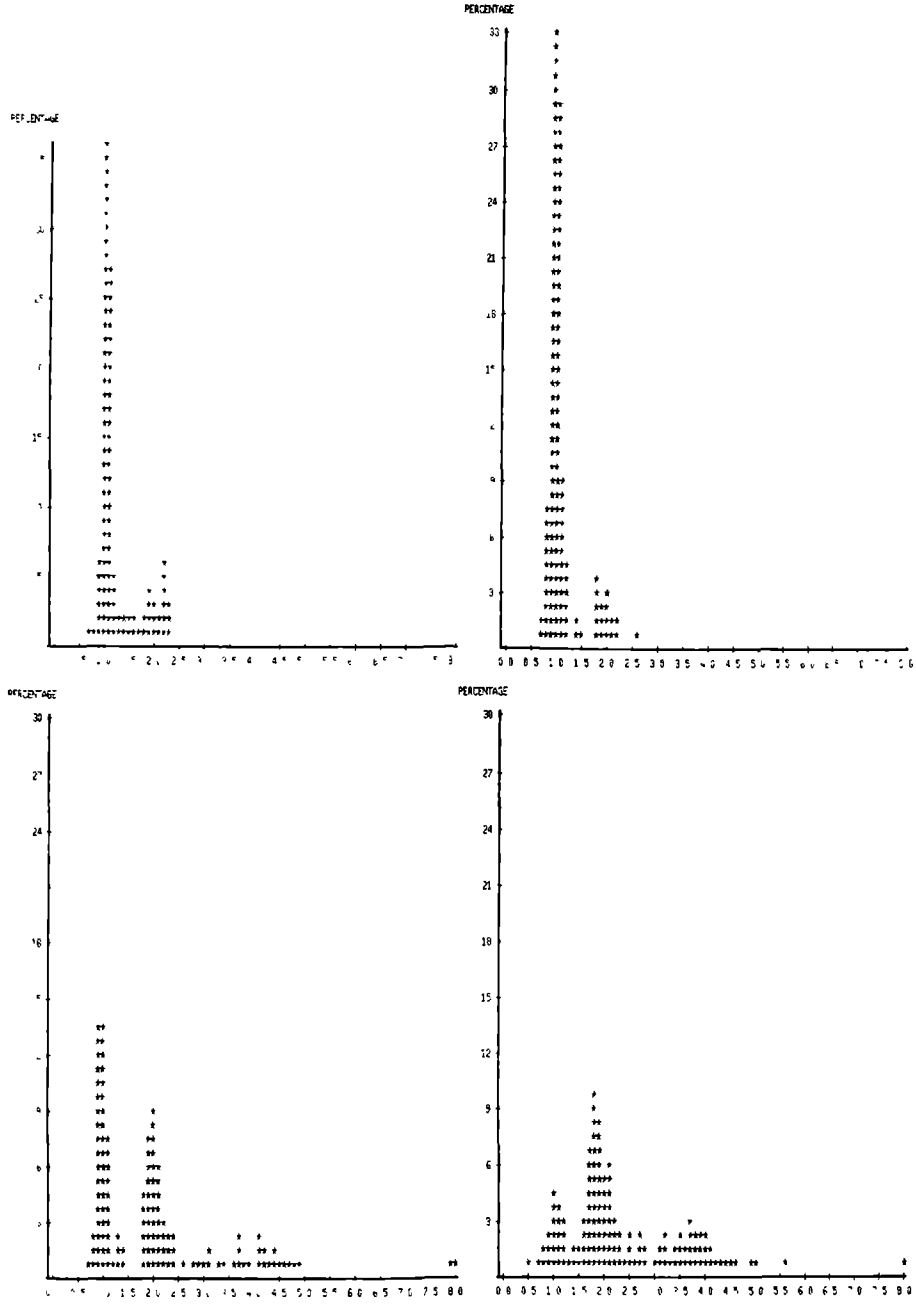

Figure 3A-3D: Diploid DNA patterns present in both (A, top left) the CIN III-like lesion (CIN.INV) and (B, top right) the adjacent invasive cancer (INV) (patient 33 years of age). (C, bottom left) Polyploid CIN III-like lesion (CIN.INV) and (D, bottom right) polyploid invasive cancer (INV) (same patient as Figs. 2C and 2D).

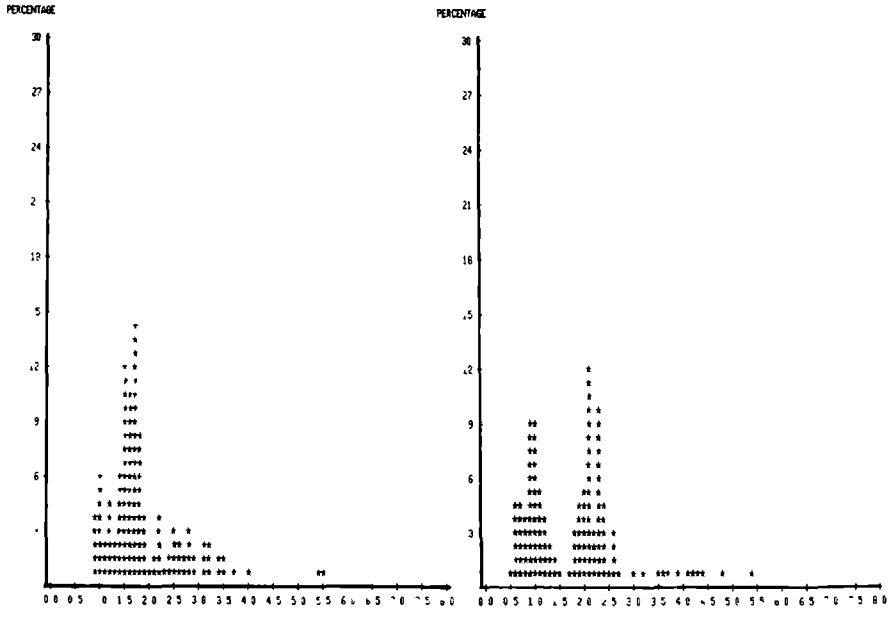

Figure 4A-4B: (A, left) Aneuploid DNA pattern of CIN III-like lesion (CIN.INV) and (B, right) polyploid DNA pattern in invasive cancer (INV) in the same patient (patient 62 years of age).

DISCUSSION

Cytophotometric and flow cytometric DNA analysis of selected areas of cervical intraepithelial neoplasia grade III and invasive squamous cell carcinoma was performed using whole nuclei extracted from paraffin-embedded tissue (29-31). With this method selected areas of archival paraffin-embedded tissues can be analyzed retrospectively.

Stromal and normal epithelium cells can be removed from the specimen under a dissecting microscope, thus reducing the mostly diploid component of benign cell populations (29), which may obscure small aneuploid fractions. The selection of areas of CIN III or invasive cancer by elimination of nonrelevant tissue segments before nuclear isolation makes DNA histograms truly representative of the epithelial lesion under study (29,30). Furthermore, DNA measurements of

intact nuclei are more reliable than measurements in nuclei in tissue sections (8,11,12,29).

In this study sufficient material was available in seven cases of invasive cancers for both cytophotometric and flow cytometric DNA analysis. The results of both measurements were comparable: a high first (diploid) peak in the FCM histograms was the result of residual lymphocytes and stromal cells still present in the sample. In this study cytophotometric DNA analysis was possible in all cases of CIN III and invasive cancer. In 18 of the 20 cases (90%) DNA patterns of CIN III-like lesions and adjacent invasive squamous cancers were similar, implying that these CIN lesions are true precursors of invasive cancer.

Cytophotometric DNA analysis of both pure CIN III and CIN III-like lesions in the presence of invasive cancer showed DNA aneuploidy in 51%, polyploidy in 23%, and diploidy in 26%. These figures differ considerably from data published by others (9-11) even when taking into account that these authors were using different methods or somewhat different definitions of aneuploidy (Table 4).

Table 4. Comparison of the current results with data from previous studies by Jakobson et al (10) and Fu et al (11).

Study by	Fu et al. (11)	Jakobsen et al. (10)	Current study
Year	1981	1983	1987
Material	Paraffin-embedded tissue 14-μm section	Fresh biopsy	Paraffin-embedded tissue: nuclei isolated from 50-μm sections
Technique of DNA analysis	Microspectrophotometry, single wavelength, plug method	Flow cytometric analysis	Cytophotometric analysis
No of patients	100	121	39
No. of CIN III	10	47	39
DNA pattern			
Diploid	0	10	10
Polyploid	0	-	9
Aneuploid	10	37	20
Age of patients	Not investigated	Not investigated	21 patients $\leq$ 35 yr 18 patients $\geq$ 50 yr

Jakobsen et al. described FCM analysis of fresh cervical biopsy material of 121 patients. From 47 cases identified as severe dysplasia or carcinoma in situ, 37 (79%) DNA histograms were aneuploid (10).

Fu et al. described the results of microspectrophotometric DNA measurements using a single wavelength plug method in 14-μm paraffin sections. In their study of 100 patients all ten cases diagnosed as CIN III were aneuploid (11). Nasiell et al. (9) used scanning microspectrophotometry for DNA measurements in cytologic preparations, defining aneuploidy by the "5-c exceeding rate". In all six cases of carcinoma in situ in their study they found a significant aneuploid cell fraction

In none of these studies age distribution of patients was taken into account In the current study 78% (N = 14) of CIN III lesions in women 50 years of age or older, with as well as without coexisting invasive cancer, were aneuploid In contrast the frequency of aneuploidy in CIN III and CIN III-like lesions in younger women was remarkably low (29%, N = 6).

These differences in frequency of aneuploidy in the two age groups may indicate two biologically different neoplastic processes. In the group of younger women a diploid DNA pattern was found in about 60% (N:6) of intraepithelial lesions with adjacent invasive cancer (CIN INV). This high percentage is at variance with data from others.

In the study of Fu et al. none of the 10 CIN III lesions was diploid (11). A diploid pattern was recorded in CIN I and II lesions only. Diploid and polyploid DNA patterns were previously thought to be found almost exclusively in reactive processes and in regressive CIN lesions (11,13,36). When Jakobsen et al. noted about 20% diploidy in CIN III lesions (10), these authors attributed this observation to the small size of the intraepithelial lesions in their study. However, Spriggs et al. using cytogenetic techniques, in specimens of carcinoma in situ, already demonstrated the modal chromosome number in 29% of cases to be in the diploid range (37). Atkin also stressed that diploid DNA patterns of CIN III lesions did not necessarily indicate good prognosis (15).

Finally, diploid DNA patterns also have been found in cervical invasive cancer and in other malignant tumors in a variety of organs such as breast, esophagus, stomach, colon, endometrium, head and neck squamous carcinomas, and ovaries (13,36). Our data strongly support the fact that a diploid DNA pattern in a CIN III lesion does not exclude progression to invasive cancer and is not necessarily indicative of a reactive or regressive change.

The significance of polyploidy in CIN III lesions is uncertain. The assumption that polyploidy indicates a reactive change is supported by the relatively high percentage (55%) of polyploid CIN III lesions in the absence of an invasive

carcinoma (CIN) in younger women and the low percentage (10%) in older women (13%) in (CIN.INV) lesions (10%), and in invasive cancer (10%).
In three of the polyploid CIN III lesions without invasive cancer (CIN), HPV common antigen was present. This supports an infectious nature of these lesions. Further behavior of the polyploid lesions is as yet unknown.
Based on our analysis of DNA ploidy in cervical intraepithelial neoplasia grade III, using nuclei isolated from paraffin-embedded tissue, the conclusion is warranted that a significant proportion of diploid CIN III lesions in younger women have invasive potential and thus a diploid DNA pattern in CIN III lesions should not automatically be considered to be an indicator of good prognosis. Our findings also suggest that processes leading to invasive cancer of the cervix may be biologically different in younger and older women. However, the current group of 39 patients is too small to draw definitive conclusions.
Further analysis using a larger group of patients should provide more detailed information on the relation between DNA ploidy patterns of CIN III lesions of the uterine cervix and their progressive or regressive behavior.

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CHAPTER 4

DNA PLOIDY AND CYTOPHOTOMETRIC ANALYSIS OF CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III AND INVASIVE SQUAMOUS CELL CARCINOMA

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Abstract Cervical Intraepithelial Neoplasia grade III (CIN III), and Squamous Cell Carcinoma (INV) were examined using DNA ploidy and cytophotometric analysis. Based on hysterectomy, exconisation and biopsy material from 69 patients in two age categories, analysis was performed in nuclei isolated from selected areas of paraffin-embedded tissue. High percentages of DNA-diploidy in INV lesions were found mainly in the group of patients, age 45 years or younger. CIN III lesions in women, age 46 or older, demonstrated high percentages of DNA-aneuploidy. DNA-polyploidy was most frequent in CIN III lesions in the younger age category. The results of cytophotometric analysis indicated that the overall mean values of 16 nuclear photometric features discriminated significantly between the complete groups of CIN III (N = 37) and INV (N = 32). On an individual patient level however the mean feature values showed a large overlap. Based on the results of a stepwise linear discriminant analysis of patient mean values, a combination of geometrical and runlength texture features was used to discriminate between CIN III and INV lesions. The correct classification rate was highest in the category of patients in the older age category. The results of this study indicate age related differences in CIN III and invasive squamous cell carcinoma. The results of this study may be of help in assessing cytophotometric features in the study of progressive and non-progressive CIN lesions.

INTRODUCTION

Cervical Intraepithelial Neoplasia grades I to III (CIN I - III = Dysplasia and Carcinoma in situ) are potentially precancerous lesions. They can progress to invasive squamous cell carcinoma, but also persist or return to normal. Earlier

20,26,28). The end point of most follow-up studies of CIN I and II, was the development of CIN III (= severe dysplasia and carcinoma in situ), at what time the patients were treated or left the study. Data on the follow-up of CIN III itself are less frequent but generally indicate that a relatively small number of CIN III lesions will eventually progress to an invasive squamous cell carcinoma (9,12,13,18, 19). Light microscopically CIN III can not be subdivided in progressive, persistent or regressive subtypes, and as a consequence all cases of CIN III are treated as essentially premalignant, potentially progressive lesions.

An alternative for visual light microscopic evaluation of CIN III lesions, is the study of nuclear DNA ploidy and cytophotometric analysis of structural nuclear changes. DNA ploidy analysis, using flow cytometric and histometric techniques, have indicated high percentages of DNA-aneuploidy in CIN III lesions (10,16). On this basis some authors concluded that DNA-aneuploidy is a marker for progression in CIN lesions (10). In a recent study on 39 patients with CIN III, with and without synchronous invasive squamous cell carcinoma, we found DNA-aneuploidy in about 50 % of cases (14). A striking observation in our study was the effect of age, dividing the group of patients in two categories: in women 50 years of age or older, a DNA-aneuploid pattern was present in 88 %, whereas in women 35 years of age or younger, a DNA-aneuploid pattern was only present in 27 %. In women 35 years or younger DNA-polyploidy was the most frequent pattern in CIN III lesions. A DNA-diploid pattern was most often found in CIN III-like lesions adjacent to invasive squamous cell carcinoma. Our findings suggested that processes finally progressing to invasive cancer of the cervix are biologically different between younger and older women. In addition to the studies of Atkin, who showed high percentages of DNA-diploidy in cervical invasive cancer, we found that a diploid DNA pattern not necessarily implied a regressive or persistent behavior of the CIN III lesions (1,2,14).

Nuclear characteristics of CIN III and Invasive Squamous cell carcinoma can also be studied by cytophotometric analysis. Digitized images offer information on nuclear features such as geometry, density and texture, that are not perceived on light microscopic evaluation even by well-trained human observers. Cytophotometric analysis was proven to be of value in the study of cervical intermediate cells present in "normal", dysplastic and carcinoma in situ specimens, in studies of normal and malignant endometrium, in the differential diagnosis in pleural

effusions and in the discrimination between classic and variant type small cell lung cancer cell lines (5,6,17,23,24).

In the present study digitized interphase nuclei of CIN III lesions and of cervical invasive squamous cell carcinomas were analyzed for DNA ploidy and cytophotometric nuclear features in an effort to characterize the pre-invasive CIN III lesions and cervical invasive squamous cell carcinomas by image analysis techniques. Furthermore it will be assessed whether the findings can be used for classification of lesions in individual patients.

In order to identify differences between regressive or persistent and progressive intraepithelial abnormalities, future analyses will deal with cytophotometric analysis of CIN III-like lesions bordering invasive squamous cell carcinoma and of CIN lesions that showed progression to invasive squamous cell carcinoma (15).

MATERIAL AND METHODS

Patient group.

Paraffin-embedded cervical tissue specimens of 69 patients, treated by hysterectomy, exconisation or extensive diathermic biopsy, were studied. In 37 patients the histological diagnosis was Cervical Intraepithelial Neoplasia grade III, without evidence of (micro-) invasive squamous cell carcinoma (CIN III). An intraepithelial lesion was defined as a CIN III lesion when "undifferentiated" basal or parabasal type cells with irregular arrangement, crowding, indistinct cytoplasmic boundaries, nuclear enlargement, a high nucleo-cytoplasmic ratio and frequent mitotic figures, occupied from two thirds up to the full thickness of the epithelial lining (7). In 32 patients the histological diagnosis was cervical invasive squamous cell carcinoma (INV). These two groups were further subdivided into age categories, 45 years of age or younger and 46 years of age or older (Table 1).

Cell material.

The specimen preparation procedure, which has been described in detail elsewhere, includes extraction of intact nuclei from selected areas of paraffin-embedded tissue (14,21,22). In short, it includes selection of the CIN III, and INV lesions from 50 micrometer "thick" paraffin-embedded tissue sections, using a dissecting

microscope. After incubation with 0.1 % protease of 37°C for 60 minutes and centrifugation, the cell suspension is placed on slides and stained with a DNA specific Thionin-Feulgen stain (23).

Table 1 Cervical epithelial abnormalities, subdivided into type of lesion and age categories *

Type of lesion	CIN III	INV	Total
Number of lesions	37	32	69
Number of patients in two age categories			
≤45 yrs	26	15	41
≥46 yrs.	11	17	28

* CIN III = cervical intraepthelial neoplasia grade III INV = invasive squamous cell carcinoma

Digitization

Approximately 200 nuclei per specimen were used for cytophotometric analysis Cell images were digitized with an Orthoplan (Leitz Wetzlar, W.-Germany) scanning microscope, provided with a linear diode array (Fairchild, USA), which was interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Manyard, MA). Nuclei were measured with a 40 x objective and monochromatic light at a wavelength of 585 nm (band width 20 nm). The sampling density was four pixels per micrometer. Nuclear boundaries were automatically determined from the digital image by grey level boundary following. As an "internal" standard for DNA-content 20 lymphocytes were measured in each of the samples. Twenty-one nuclear features, describing geometrical, density and texture characteristics (Table 2) and one sample feature (total DNA content in the total sample of nuclei) were extracted from the digitized image (24) DNA content of measured nuclei was expressed as DNA-index (D I.), defined as the integrated optical density of a nucleus divided by the median integrated optical density of the "internal" control lymphocytes (14,22,23). D I distributions were estimated by frequency histograms of a sample of nuclei, using a bin-size of 0.1. These DNA ploidy patterns were visually classified as DNA-diploid, DNA-polyploid, or DNA-aneuploid, according to the following definitions. In a DNA-diploid pattern a distinct G0/G1 peak is found in the (near-)diploid (2C, D I. = 1.0 +/- 10 %) region with a small proportion of cells in S and G2/M (4C) mode, as in

normal tissue. In DNA-polyploidy distinct peaks were present in the diploid (2C; D.I. = 1.0 +/- 10 %) and tetraploid (4C; D.I. = 2.0 +/- 10 %) regions, or in the diploid, tetraploid and octaploid (8C, D.I. = 4.0 +/- 10 %) regions. Ploidy patterns were considered DNA-aneuploid in all cases with scattered D.I. distributions, or uni-, bi-, or multimodal D.I. distributions, that were non-diploid and non-polyploid

Table 2 List of photometrical nuclear features

TEXTURE	
Runlength	
1 SRL	Short Runlength Emphasis
2 LRL	Long Runlength Emphasis
3 GLD	Greylevel Distribution
4 RLD	Runlength Distribution
5 RPC	Run Percentage
Cooccurrency	
6 HOMOG	Homogeneity
7 CONTR	Contrast
8 INVCON	Inverse Contrast
9 ENTRO	Entropy
10 MXTRP	Maximum Transition Probability
11 DMOM1	First Diagonal Moment
12 DMOM2	Second Diagonal Moment
13 TRISY	Triangular Symmetry
DENSITY	
14 DI	DNA Index
15 ADI	Average DNA Index
16 SDDI	Standard Deviation of DNA Index
17 M3DI	Third Moment of DNA Index
GEOMETRY	
18 AREA	2-D Area of the nucleus
19 CIRC	Circularity
20 GMRAT	Geometrical Moments Ratio
21 MASSEC	Mass Eccentricity, DNA margination

Cytophotometric analysis of 21 nuclear features was performed on a total of 12562 cell nuclei of 69 lesions. First the mean values of nuclear features in the complete groups of CIN III (n = 37) and INV (n = 32) lesions were calculated

Thereafter the mean values and standard deviations of the nuclear features in all individual patients were calculated and used in a statistical analysis to determine the most promising feature for classification purpose.

Statistical Analysis.

Statistical analysis on the sets of 21 photometric nuclear features was carried out using the Statistical Analysis System package (29), e.g. general linear models procedure, stepwise linear discriminant analysis and linear discriminant analysis. The general linear models procedure was used to characterize the groups of CIN III and INV. A stepwise linear discriminant analysis, using the data from 21 photometric features obtained from 69 patients subdivided in CIN III and INV groups, was used to select the best discriminating parameters and to construct a classification criterium for patients with CIN III and INV lesions. This classification was performed by linear discriminant analysis.

RESULTS

DNA ploidy pattern analysis.

The results of visual interpretation of DNA ploidy patterns of 37 CIN III lesions and 32 INV lesions are shown in table 3. A DNA-diploid pattern was found more frequent in INV lesions (15 of 32 cases, 47 %) than in CIN III lesions (9/37 cases, 24%). A DNA-polyploid pattern was found more frequent in CIN III cases (13/37 cases, 35 %), than in INV lesions (5/32 cases, 16 %) lesions. The frequency of DNA-aneuploidy was slightly but not significantly higher in CIN III than in INV lesions; 41 % (15/37) in CIN III and 37 % 12/32) in INV lesions. The distribution of DNA ploidy patterns in CIN III and INV showed distinct differences between the two age categories e.g. patients 45 years of age or younger and patients 46 years of age or older (table 3). DNA-aneuploidy was found in 23 % of CIN III lesions and 20 % of INV lesions in the younger age group, and in 82 % of CIN III lesions and in 53 % of INV lesions in the older age group. A DNA-diploid pattern was found in the younger age group in 31 % in CIN III lesions and in 73 % in INV lesions, and in the older age group in 9 % of CIN III cases and in 24 % of INV lesions.

Table 3 DNA ploidy patterns of CIN III and INV Total and subdivided for two age categories

DNA ploidy pattern	Total		≤ 45 yr		≥ 46 yr	
	N	%	N	%	N	%
CIN III						
Diploid	9	24	8	31	1	9
Polyploid	13	35	12	46	1	9
Aneuploid	15	41	6	23	9	82
Total	37	100	26	100	11	100
INV						
Diploid	15	47	11	73	4	24
Polyploid	5	16	1	7	4	24
Aneuploid	12	37	3	20	9	53
Total	32	100	15	100	17	101

A DNA-polyploid pattern was most frequently found in CIN III lesions in the younger age category (46 %) In the older age category in CIN III lesions this was 9 %. DNA-polyploidy in INV-lesions was present in 7 % in the younger group and in 24 % in the older age category.

Cytophotometric analysis.

The mean values of the nuclear features in the groups of CIN III and INV are shown in table 4. Using the general linear models procedure a significant discrimination between the groups of CIN III and INV could be made by the group mean values of 16 of the 21 nuclear features (SRL, LRL, GLD, RPC, HOMOG, CONTR, INVCON, ENTRO, MXTRP, DMOM1, DMOM2, DI, ADI, CIRC, GMRAT, and MASSEC). In table 4 highest values of the significantly different features are underlined. Group mean values of seven nuclear features (SRL, RPC, CONTR, ENTRO, DMOM1, DMOM2, and CIRC) were significantly higher in INV than in CIN III lesions. Group mean values of nine other features (LRL, GLD, HOMOG, INVCON, MXTRP, DI, ADI, GMRAT, and MASSEC) were significantly higher in CIN III than in INV lesions. On individual patient level the mean feature values showed a large overlap

Table 4 Mean values of nuclear photometric features in CIN III and INV lesions The highest values of significantly different features, (P < 0 05) are underlined.

Features	CIN III	INV
Texture, runlength		
1 SRL	0 277	0 300
2 LRL	97 0	87 8
3 GLD	0 185	0.177
4 RLD	0 145	0 148
5 RPC	0 158	0 169
Texture, co-occurency		
6 HOMOG	0 0457	0 0438
7 CONTR	1 065	1 136
8 INVCON	0 699	0 688
9 ENTRO	1 441	1 455
10 MXTRP	0 0983	0 0971
11 DMOM1	3 304	3 420
12 DMOM2	0 338	0 354
13 TRISY	0 103	0 101
Density		
14 DI	1 645	1 570
15 ADI	0 000909	0 000825
16 SDDI	0 000346	0 000344
17 M3DI	0 000690	0 000681
Geometry		
18 AREA	1905	1929
19 CIRC	0 770	0 774
20 GMRAT	2 62	2 38
21 MASSEC	0 451	0 447

The results of stepwise linear discriminant analysis, using the mean (M) feature values of individual patients, indicated that the most promising parameters for discrimination between CIN III and INV were two geometrical and two runlength texture features (in order of significance: M-GMRAT, M-SRL, M-MASSEC and M-LRL). With these features 78% of the CIN III and INV lesions could be classified correctly in individual patients. The results of linear discriminant analysis are presented in table 5-A. The false positive rate, defined as the percentage of "a priori" CIN III lesions classified as INV, was 22 %. The missed-positive rate, defined as the percentage of "a priori" INV lesions classified as CIN III was also 22 %.

Table 5: Cytophotometric analysis; classification results of discriminant analysis between CIN III and INV lesions, based on four features, M-GMRAT, M-SRL, M-MASSEC and M-LRL. (Total and in two age categories).

	CIN III		INV		Total	
	N	%	N	%	N	%
A: Total groups of CIN III and INV.						
CIN III	29	78	8	22	37	100
INV	7	22	25	78	32	100
Total	36	52	33	48	69	100
B: Lesions in women ≤ 45 years of age.						
CIN III	20	77	6	23	26	100
INV	6	40	9	60	15	100
Total	26	63	15	37	41	100
C: Lesions in women ≥ 46 years of age.						
CIN III	10	91	1	9	11	100
INV	1	6	16	94	17	100
Total	11	39	17	61	28	100

The morphologic picture that emerges from these analyses is that the nuclei of CIN III lesions have a less rounded, more elongated shape, more DNA margination more coarse and less fine granularity of the chromatin than the nuclei of INV cells. When included in a stepwise linear discriminant analysis, numerical age was the second most important parameter, next to feature M-GMRAT. This could be partly explained by the number of younger patients with CIN III lesions, in this study, being more than twice the number of older patients with CIN III lesions. A linear discriminant analysis using age as a numerical parameter was therefore not performed for the entire series of patients. For each separate age category (45 or younger and 46 or older) however a linear discriminant analysis of CIN III and INV, was possible. This analysis, based on the features M-GMRAT, M-SRL, M-MASSEC and M-LRL, showed distinct patterns for the two age categories, as illustrated in tables 5-B and 5-C. In the youngest age category a correct classification of CIN III was made in 77 % (false positive rate of 23 %) and of INV in 60 % (false negative rate of 40 %). In the oldest age category, the correct classification of CIN III was 91 % (false positive rate of 9 %) and the correct

classification of INV was 94 % (false negative rate of 6 %).
The results of stepwise linear discriminant analysis, including standard deviations (S) of the feature values of individual patients demonstrated a slight shift of the most significantly discriminating features. The most promising features were M-GMRAT, M-SRL, S-CONTR and M-LRL. With those features the linear discriminant analysis of CIN III and INV, gave similar results as was found using only the mean values of the cytophotometric features. The correct classification of the complete groups of CIN III and INV were 78% and 75% respectively. In the younger age category these percentages were 69% and 68%; in the older age category they were 91% and 88% respectively. A Jackknife procedure, performed on these data using the latter four features showed a 76% correct classification in the complete group of CIN III lesions and 75% correct classification in the complete group of INV lesions.

DISCUSSION

DNA ploidy pattern analysis of 37 cervical intraepithelial neoplasias grade III and 32 invasive squamous cell carcinoma lesions showed that there is not a single specific DNA ploidy pattern that can characterize the group of CIN III or INV lesions. The present results lent support to earlier findings that DNA-diploidy does occur in CIN III and INV lesions and therefore that DNA diploidy does not exclude an invasive potential (1,14). This is consistent with the finding that DNA-diploidy, as measured by DNA ploidy analysis, not automatically means that a complete set of 46 normal chromosomes is present in each measured cell. Structural chromosome changes and small numeric changes will not be discovered by DNA ploidy analysis alone. The present study of DNA ploidy patterns of 69 specimen of CIN III and INV lesions does indicate age related differences. DNA-diploidy was encountered most often in INV (73 %) lesions in patients, 45 years or younger. The highest frequency of DNA-polyploidy was found in CIN III lesions in this age category (46 %). DNA-aneuploidy was most frequent in CIN III (82%), and INV (53%) lesions in women 46 years of age or older. With increasing age DNA ploidy patterns of these lesions tend to be more frequently aneuploid. This raised the question whether DNA-aneuploidy is more age related than tumor related and whether DNA-aneuploidy could also be found in normal cervical tissue of older women. However, in a pilot series of formalin fixed, paraffin-embedded,

normal ectocervical epithelium from ten women 46 years of age or older, using the same preparation technique as used in the present study, all specimen were DNA-diploid. No DNA-aneuploidy was found.

In a study of DNA ploidy in cervical squamous cell carcinoma, Atkin also noted differences in age distribution according to the ploidy group. He described a possible tendency for younger patients (up to 40 years) to have near-diploid and older patients (over 70) to have near-triploid or near-tetraploid tumors (1). The mean age of patients at the time of detection of an invasive squamous cell carcinoma of the cervix is around 50 years (48.2-53.4 years) (25,26). The mean age of patients with diagnosis of carcinoma in situ is around 40 years (36.6-42.5 years) (25,26). Recently carcinoma in situ was reported to be diagnosed with an increased frequency in women under the age of 35 (11). Furthermore Berkeley et al and Prendiville et al reported the occurrence of cervical invasive carcinoma in young women after a recent negative cervical cytologic control, indicating that cervical cancer in young women may have a very short preinvasive phase (3,27). Nasiell et al observed, in a follow-up study of 849 patients with moderate dysplasia (CIN II), different progression rates in women over 51 and in younger women (20). In women over 51 years of age the progression interval time was significantly longer than for women in the age group of 26 to 50 (20). In view of these and our present findings we strongly doubt if a new classification of Cervical Intraepithelial Neoplasias should be based exclusively on ploidy patterns, as was suggested by Bibbo et al (4). In our study the differences of DNA ploidy patterns between CIN III and INV lesions were small, with a relatively high number of lesions in some of the age groups demonstrating a DNA diploid pattern.

Through the use of cytophotometric technique, as performed in this study, CIN III lesions and invasive (INV) lesions could be characterized by 16 of 21 nuclear features. With the use of mean values of four nuclear features, CIN III and INV lesions were correctly classified in 78 % of cases. Some authors state that cytological specimens of in situ carcinoma and invasive carcinoma cannot be discriminated on a cellular basis alone (25). According to others invasive carcinoma can be discriminated from carcinoma in situ by the presence or absence of macronucleoli in the nuclei in invasive carcinoma (25). Our present findings of the significance of Mass Eccentricity of the nuclear chromatin in the discrimination of CIN III and INV are consistent with earlier cytologic finding of better defined nuclear membranes in carcinoma in situ, when compared with invasive carcinoma. In the present study the shape in CIN III nuclei was found to be more elongated and less round,

based on features GMRAT and CIRC. This is not completely consistent with findings of Patten, who described a slightly higher frequency of rounded nuclei in carcinoma in situ (25). Our finding of the discriminating power of Short and Long Runlength Emphasis correspond with the light microscopic observation of a more coarse granular chromatin pattern in carcinoma in situ when compared with invasive carcinoma (25). As could be expected the mean area of CIN III nuclei was lower than that of INV lesions, however not significantly so, because of considerable overlap between patients.

Our finding of the discriminating power of cytophotometric chromatin texture and geometrical nuclear features, correlate with previous findings in cervical intermediate cells, normal and malignant endometrium, lymphocytes, pleural smears and small cell lung cancer cell lines (4,5,6,17,23,24). The significance of the subdivision in two age categories was illustrated by the effect of age on the correct classification rate of CIN III and INV, being 77 % and 60 % respectively in the youngest age category and 91 % and 94 % in the oldest age category. The cytophotometric and DNA ploidy analyses in this study indicate that age could be an important parameter in the study of nuclear DNA content and distribution. These differences may be explained by different underlying biological processes, related to CIN III lesions and invasive squamous cell carcinomas in younger and in older women. There may be the effect of different etiological moments (like repeated viral infections), but it may also be an effect of different types of tissue reaction in the cervix uteri in patients in these age categories.

Based on the results of the present study, it may be concluded that cytophotometric analysis distinguishes between CIN III and INV lesions in nuclei retrieved from paraffin embedded lesions. Furthermore cytophotometric analysis proved to be a better technique for discrimination between CIN III lesions and INV lesions, than DNA ploidy analysis. Whether cytophotometric analysis can be of value in discriminating between progressive and regressive CIN lesions, needs to be further evaluated. The present results will be used as a model system for a future analysis of Cervical Intraepithelial Neoplasia grade III - like lesions related to synchronous invasive squamous cell carcinoma and of CIN I, II and III lesions in patients, which after a long interval period, showed progression to invasive squamous cell carcinoma.

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CHAPTER 5

CYTOPHOTOMETRIC ANALYSIS OF CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III, WITH AND WITHOUT SYNCHRONOUS INVASIVE SQUAMOUS CELL CARCINOMA

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CYTOPHOTOMETRIC ANALYSIS OF CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III, WITH AND WITHOUT SYNCHRONOUS INVASIVE SQUAMOUS CELL CARCINOMA

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Abstract Cytophotometric analysis was performed in nuclei retrieved from paraffin-embedded cervical tissue from 57 cases of CIN III. CIN III lesions of patients without invasive squamous cell carcinoma (N=37), were regarded to represent a mixture of progressive and non-progressive lesions. The CIN III lesions of patients with a synchronous invasive squamous cell carcinoma (N=20), were regarded as representing truly progressive precursor lesions (CIN.INV). Twenty-one photometric features describing geometrical, density and texture characteristics were extracted from the digitized nuclear images. Statistical analysis of cytophotometric data indicated significant differences between the group of CIN III lesions and CIN.INV lesions. A cluster analysis, using one co-occurrency texture feature (S-HOMOG), one density feature (S-DI) and two geometrical features (S-AREA and M-CIRC), showed that two clusters (C1 and C2) were present in the total group of CIN III and CIN.INV lesions. The vast majority of CIN.INV lesions was member of one and the same cluster C1. The CIN III group appeared to consist of a mixture of two clusters, 54% C1 and 46% C2 lesions. Of patients 45 years or younger, the majority (62%) of CIN III lesions had feature values, corresponding with those of cluster C1, and as such possibly with a potentially progressive course. In patients older than 45 years the percentage of CIN III lesions with C1 feature values was 27%.

INTRODUCTION

Patients with Cervical Intraepithelial Neoplastic (CIN) lesions are more and more

treated in an early stage of disease by destructive surgical methods such as diathermic ablatio, laserevaporisation, cryocoagulation, exconisation or even hysterectomy. Although cervical intraepithelial neoplasia lesions may progress to invasive cancer, a significant part would not have a progressive course when left untreated (6,12,13,16,22). The precise frequency of progression of CIN into invasive squamous cell carcinoma is not known.

It is not possible to predict by visual lightmicroscopic observations alone, what CIN lesions will have a progressive or a non-progressive course. Therefore light microscopically detected CIN I - III lesions must be regarded as a mixture of potentially progressive, persistent and regressive lesions. A logical approach to the study of progressive CIN lesions is to study those CIN III lesions, that are found adjacent to invasive squamous cell carcinoma. The study of CIN III lesions adjacent to invasive carcinomas is not new; it was already in 1910 that Rubin speculated that the epithelial abnormalities adjacent to invasive carcinomas were the preinvasive stages of cancer (25). CIN III lesions that are adjacent to invasive carcinoma (CIN.INV) cannot be discriminated from CIN III lesions without adjacent invasive cancer (CIN III), by lightmicroscopical inspection.

An alternative approach for the lightmicroscopical evaluation of CIN III lesions, is cytophotometric analysis of nuclei, stained with a DNA specific stain (1,3,9,11). Measurement of texture, density and geometry of digitized nuclei is objective and allows statistical analysis. In a recent cytophotometric study of nuclei isolated from paraffin-embedded tissue we indicated the importance of this technique in discriminating between CIN III and invasive squamous cell carcinoma (9). It was possible to differentiate between nuclei derived from CIN III lesions and nuclei from invasive squamous cell carcinoma (INV) by the group mean values of 16 nuclear features and to classify individual lesions correctly in 78% of cases by the patient mean values of two geometrical and two texture features. The results of classification varied in two age categories. The correct classification rates were lowest in the group of women 45 years of age or younger (77% correct classification in CIN III and 60% in INV) and highest in the group of women 46 years of age or older (91% correct classification in CIN III and 94% in INV).

In the present study a cytophotometric analysis of texture, density and geometrical features was performed in digitized interphase nuclei from CIN.INV and CIN III lesions. The premalignant potential of CIN III lesions without concomitant invasive squamous cell carcinoma was regarded as "unknown", which implies that in this group of lesions potentially progressive and non-progressive lesions were thought

to be present. CIN.INV lesions with synchronous invasive squamous cell carcinoma were regarded to represent truly progressive precursor lesions (8).
The aim of the study was to find features discriminating between progressive and regressive or persistent CIN III lesions.

MATERIAL AND METHODS

Patient groups.

Paraffin-embedded cervical tissue derived from hysterectomy or exconisation specimens and from biopsy material of 57 patients was studied. In 37 patients the histological diagnosis was Cervical Intraepithelial Neoplasia grade III, without concomitant invasive squamous cell carcinoma (CIN III) (Table 1).

Table 1: Cervical intraepithelial abnormalities, subdivided by type of lesion and age categories.

Type of lesion *)	CIN III	CIN.INV	Total
Patients			
<= 45 yrs.	26	10	36
> 45 yrs.	11	10	21
Total	37	20	57

* CIN III = Cervical Intraepthelial Neoplasia Grade III. CIN.INV = Cervical Intraepthelial Neoplasia Grade III, adjacent to Invasive Squamous Cell Carcinoma.

In 20 patients the histological diagnosis was Cervical Intraepithelial Neoplasia grade III with concomitant cervical invasive squamous cell carcinoma (denoted as CIN.INV and INV respectively). An intraepithelial lesion was defined as CIN III or CIN.INV, when "undifferentiated" basal or parabasal type cells, with nuclear enlargement, indistinct cell boundaries, a high nucleo-cytoplasmic ratio and frequent mitotic figures occupied from two thirds up to the full thickness of the epithelial lining (5). These two groups of patients were further subdivided into two age categories; 45 years of age or younger (N = 36) and older than 45 years of age (N = 21) (9).

Cell material.

The specimen preparation procedure included selection of CIN III, CIN.INV and INV areas in 50 micrometer, "thick" paraffin-embedded tissue sections, using a dissecting microscope (8,17,18). After incubation with 0.1% protease of 37 C for 60 minutes and centrifugation, the cell-nuclei were transferred to slides and stained with the DNA specific Thionin-Feulgen stain (19).

Measurements

Per specimen about 200 nuclei were digitized using an Orthoplan (Leitz Wetzlar, W.-Germany) scanning microscope, provided with a linear diode array camera (Fairchild, USA), which was interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Manyard, MA). Nuclei were measured with a 40 x objective and monochromatic light at a wavelength of 585 nm (band width 20 nm). The sampling density was four pixels per micrometer. Nuclear boundaries were automatically determined from the digital image by grey level boundary following. Twenty-one nuclear features, describing geometrical, density and texture characteristics were extracted from the digitized image (table 2) (7,8,10,20,21,24). As an internal standard for DNA-content 20 lymphocytes were measured in each of the samples. Mean (M) and standard deviation (S) values of the 21 nuclear photometrical features were calculated for each lesion.

Statistical Analysis

The aim of the study was to determine if the group of CIN III lesions could be subdivided into two subgroups: 1) a group of lesions with similar feature values to the group of CIN.INV lesions and as such possibly representing potentially progressive lesions, 2) a group of lesions with feature values dissimilar to the CIN.INV lesions. For this purpose features were selected that could discriminate between CIN III and CIN.INV lesions, assuming these to be good candidates for discrimination of the two subgroups. Depending on the distribution of the features a t-test or Mann-Whitney U-test were used to select features discriminating between the groups of CIN III and CIN.INV.

Table 2 List of photometrical nuclear features.

TEXTURE	
Runlength	
1:SRL	Short Runlength Emphasis
2:LRL	Long Runlength Emphasis
3:GLD	Greylevel Distribution
4 RLD	Runlength Distribution
5 RPC	Run Percentage
Cooccurrency	
6 HOMOG	Homogeneity
7.CONTR	Contrast
8·INVCON	Inverse Contrast
9 ENTRO	Entropy
10 MXTRP	Maximum Transition Probability
11 DMOM1	First Diagonal Moment
12 DMOM2	Second Diagonal Moment
13 TRISY	Triangular Symmetry
DENSITY	
14 DI	DNA Index
15:ADI	Average DNA Index
16 SDDI	Standard Deviation of DNA Index
17 M3DI	Third Moment of DNA Index
GEOMETRY	
18 AREA	2-D Area of the nucleus
19 CIRC	Circularity
20 GMRAT	Geometrical Moments Ratio
21 MASSEC	Mass Eccentricity; DNA margination

A stepwise linear discriminant analysis was performed, based on all 42 M and S feature values (after an adequate transformation to "normality") of individual patients. All selection procedures were performed in the complete groups of CIN III and CIN.INV lesions and in lesions of women in two age categories, viz. <=45 years of age and > 45 years.

Ward's cluster analysis was carried out within the total group of lesions, using the best discriminating M and S features from the t-test, Mann Whitney U-test and stepwise discriminant procedures (26,27). The Ward procedure implies the use of standard deviation-scores of the features. The clustering procedure aimed to reach a division of the CIN III group under the condition that the CIN.INV

group was not splitted up. In this manner CIN III lesions could be identified, that had corresponding feature values to the CIN.INV group. Due to the small numbers of observations, the results of the procedures for feature selection and cluster analysis must be regarded as explorative.

RESULTS

Selection of features in CIN III and CIN.INV lesions.

To select features for the cluster analysis a t-test or Mann-Whitney U-test and a stepwise discriminant analysis were performed. Using the t-test a significant difference ($p<0.05$) between the complete groups of CIN III and CIN.INV was found for the mean and standard deviation of DNA Index (M-DI,S-DI) and for the standard deviation of Area (S-AREA). In the older age group significant differences between CIN III and CIN.INV were found for the standard deviation of one texture feature (S-HOMOG), the standard deviation values of three density features (S-DI, S-ADI, S-SDDI) and the standard deviation and mean of two geometrical features (M-CIRC and S-AREA). In the younger age group the mean value of circularity (M-CIRC) was the only parameter that differed significantly between CIN III and CIN.INV.
The stepwise discriminant analysis (p-entry <0.05) of the complete groups of CIN III and CIN.INV, using the mean and standard deviation feature values of individual patients yielded one discriminative feature; S-DI. In a stepwise discriminant analysis in patients $<=$ 45 years of age, the most promising features were S-DI and M-CIRC. The stepwise discriminant analysis performed in the group of patients older than 45 years identified as the most promising features S-HOMOG, M-AREA and S-LRL. The feature S-DI, that was initially selected as the most promising feature, was discarded during the procedure and replaced by S-LRL.

Cluster analysis of CIN III and CIN.INV.

As variables in the cluster analysis those features were chosen, that were selected twice or more during the above mentioned procedures for the discrimination between CIN III and CIN.INV. These variables included the standard deviation

of one texture feature (S-HOMOG), the standard deviation of one density feature (S-DI), the standard deviation (S-AREA) and mean value (M-CIRC) of two geometrical features.
The results of the cluster analysis are presented in table 3.

Table 3: Cluster analysis of CIN III and CIN.INV, based on S-HOMOG, S-DI, S-AREA and M-CIRC. A: complete groups of patients, B: patients <= 45 years, C: patients > 45 years.

A: Complete groups of CIN III and CIN.INV.			
	Cluster 1	Cluster 2	Total
CIN III	20 (54%)	17 (46%)	37 (100%)
CIN.INV	18 (90%)	2 (10%)	20 (100%)
B: CIN III and CIN.INV in patients <= 45 years.			
	Cluster 1	Cluster 2	Total
CIN III	16 (62%)	10 (38%)	26 (100%)
CIN.INV	10 (100%)	-	10 (100%)
C: CIN III and CIN.INV in patients > 45 years.			
	Cluster 1	Cluster 2	Total
CIN III	3 (27%)	8 (73%)	11 (100%)
CIN.INV	8 (80%)	2 (20%)	10 (100%)

First the total groups, irrespective of age were analyzed (Table 3-A). The vast majority (90%) of CIN.INV lesions appeared to belong to the same cluster (cluster 1). According to our hypothesis this cluster would consist of the potentially progressive lesions. Two cases of the CIN.INV lesions were found in cluster 2. About half of the CIN III lesions (20 cases, 54%) were member of the same cluster 1. The remaining CIN III lesions (17 cases, 46%) were found to make up the second cluster.
In table 3-B the clustering results for the younger age category are represented. All of the CIN.INV lesions of patients in the age category <= 45 years, were a member of cluster 1. In this age group, the number of CIN III lesions belonging to cluster 1, was higher (16 cases, 62%) than the number of CIN III belonging to cluster 2 (10 cases, 38%).
In table 3-C the clustering results for the older age category are represented. Eight of the CIN.INV lesions were a member of cluster 1 (80%). The two CIN.INV lesions that were a member of cluster 2 in the cluster analysis of the

complete groups, also belonged to the second cluster of the cluster analysis in this specific age group. In one of these cases the CIN.INV lesion was located at an uncommonly large distance from the invasive cancer region. Therefore it was possible that this lesion that was introduced as a CIN.INV lesion, in fact was a synchronous CIN III lesion of non-progressive potential. The second case showed a relatively high S-HOMOG value. Further analysis of this case showed a very low mean and standard deviation of integrated optical density of the cytospin specimen. This may have influenced the texture measurements, due to noise enhancement by equalization of "flat" (non-textured) nuclei (21). The results of cluster analysis of CIN III lesions in the older age group demonstrated a lower number of lesions belonging to cluster 1 (3 cases, 27%), than to cluster 2 (8 cases, 73%).

The distribution over the clusters in the complete groups was consistent with the results of cluster analysis as was performed separately in the two age groups. Only one CIN III lesion was reassigned from cluster 1 to cluster 2 as a result of these repeated cluster analysis within the subgroups.

In table 4 the means of the clustering variables of the detected clusters are presented. This table indicates the differences of the four cluster variables between the clusters 1 and 2. The feature values of cluster 1 were lower for S-HOMOG, S-DI, S-AREA and higher for M-CIRC.

Table 4 Cluster means (M) and standard deviations (S) of features used in the cluster analysis

	Cluster 1 (N = 38)		Cluster 2 (N = 19)	
	M	S	M	S
S-HOMOG	0 0055	0 0006	0 0068	0 0008
S-DI	0 491	0 19	0 844	0 13
S-AREA	526	170	866	170
M-CIRC	0 774	0 03	0 752	0 02

The morphological translation of these findings is that cluster 1 lesions have less variation in nuclear DNA-distribution, DNA-content and nuclear size, and have, on the average, more round nuclei.

Comparison with synchronous INV lesions.

In 17 CIN.INV lesions sufficient material of the synchronous invasive squamous cell carcinoma lesions was present for cytophotometric analysis. To compare these INV lesions with the cluster 1 and cluster 2 lesions, they were classified using a discriminant function as obtained by linear discriminant analysis of the clusters 1 and 2, based on the features (S-HOMOG, S-DI, S-AREA and M-CIRC) that were used for the cluster analysis.
The results demonstrated that 13 INV lesions (76%) had feature values similar to cluster 1 and four lesions had values similar to cluster 2 (24%). It was thought that INV lesions would have had cluster 1 feature values, representing a possibly progressive potential. The presence of four INV lesions with values corresponding with cluster 2, indicated that the cluster analysis of CIN III and CIN.INV was not completely applicable for INV lesions. As a tentative explanation it was considered that the final proces of infiltration that discriminates between CIN.INV and INV may involve other nuclear changes, than those that were described by the four features (S-HOMOG, S-DI, S-AREA and M-CIRC) used in the cluster analysis of CIN III and CIN.INV. A stepwise discriminant analysis performed on the complete groups of CIN.INV and INV lesions rendered a completely different group of 7 significant features. The four most promising features (M-GMRAT, M-SRL, S-GLD and M-GLD) gave a correct classification of CIN.INV in 14 of 20 cases (70%). The correct classification of the 17 cases of INV lesions was one hundred percent.

DISCUSSION

Earlier cytophotometric studies of CIN lesions concentrated mostly on features that could discriminate between, 1) normal and abnormal cell specimen, 2) CIN II specimen and CIN III or invasive carcinoma specimen, 3) intermediate cells of "normal" cytological specimen and normal appearing intermediate cells in cytological specimen with concomitant dysplastic cells (3,4,9,11,14,15,28,29,30,31). Wheeler and coworkers were able to correctly classify cells from CIN II, CIN III and Invasive carcinoma lesions in about 90% of cases, using cellular and nuclear geometrical, density and texture features (30). Recently a karyometric analysis of formalin fixed, paraffin-embedded 6 micrometer tissue sections, representing

cervical carcinoma in situ and adjacent normal ectocervical mucosa of five patients, was presented by Montag et al. They showed differences in nuclear shape and chromatin distribution of the normal appearing cervical mucosa, depending on the distance to the CIS area (15). Bibbo and coworkers presented a related study of 6 micrometer sections of four patients with cervical invasive carcinoma with adjacent normal appearing epithelium and five control patients. Among others the most important parameters for discriminating purposes were the roundness of the nucleus, the nuclear perimeter, total optical density and runlength texture measure. We were not able to find earlier reports of cytophotometric analyses of CIN III and CIN.INV lesions. Our present study of 57 patients showed that nuclei from CIN III and CIN.INV lesions yielded cytophotometric information that possibly could be of use for the discrimination between progressive and non-progressive CIN lesions. Using a cluster analysis, based on one co-occurrency texture feature (S-HOMOG), one density feature (S-DI) and two geometrical features (S-AREA and M-CIRC), we showed that the vast majority of CIN.INV lesions were part of one cluster. If indeed CIN.INV lesions are truly progressive precursor lesions as we have assumed, these findings could possibly indicate that lesions with "cluster 1" feature values may have the potential to progress into invasive lesions.

As a result of the cluster analysis in this study 20 CIN III lesions (54%) were classified as member of "cluster 1", and as such had photometrical characteristics similar to the vast majority of the CIN.INV lesions. It was of interest to observe that in women 45 years or younger, 62% of the CIN III lesions were classified as cluster 1 lesions, and in the group of patients older than 45 years this percentage was only 27%. These findings might possibly indicate that elderly women have less frequently potentially progressive CIN III lesions. The present results are difficult to compare with previous studies, since generally accepted rates of progression or non-progression of CIN III lesions are still not known (12,13,22). The present findings of a possibly more aggressive potential of CIN III lesions in younger women link up to earlier findings of Berkeley et al and Prendiville et al, who reported fast growing cervical invasive carcinomas in young women after recent negative cervical cytological controls (2,23). Furthermore in a follow-up study of 849 women with moderate dysplasia (CIN II), Nassiell et al observed that CIN II lesions in women over 51 years of age had a significantly longer progression interval time than women in the age group of 26 to 50 (16).

Four of the 17 synchronous invasive carcinoma lesions did not have similar

feature values as cluster 1. One may speculate that this celbiologically could be explained by assuming that in the final proces of infiltration, that discriminates between CIN.INV and INV, other nuclear DNA-related changes occur. In that respect it was noteworthy that a linear discrimination analysis of CIN.INV and INV rendered a 100% correct classification of INV, when four other features were used. It is of interest that the major discriminative features M-GMRAT and M-SRL were also the most discriminative features in our previous study of CIN III and INV lesions (9).

Based on the present study it is concluded that: 1) cytophotometric analysis of nuclei, retrieved from paraffin-embedded CIN III and CIN.INV lesions is possible, 2) in the group of CIN III lesions a subgroup of lesions was present that had similar photometric feature values as the vast majority of CIN.INV lesions, which as such could eventually indicate a progressive potential, 3) the majority of CIN III lesions in younger women had similar feature values as the vast majority of CIN.INV lesions. Further analysis of independent data is necessary to validate the findings of this study. The present findings are actually being used as a model system for an analysis of cytologically detected CIN lesions in patients who after a long interval period showed progression to invasive squamous cell carcinoma.

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CHAPTER 6

CYTOPHOTOMETRIC ANALYSIS OF CORRESPONDING CYTOLOGICAL AND HISTOLOGICAL CIN III SPECIMEN

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CYTOPHOTOMETRIC ANALYSIS OF CORRESPONDING CYTOLOGICAL AND HISTOLOGICAL CIN III SPECIMEN

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Abstract Cytophotometric analysis of Cervical Intraepithelial Neoplasia grade III (CIN III) was performed in 22 cytological smears (CS) and 22 corresponding cytospin specimen, retrieved from selected areas of paraffin-embedded tissues (PEC). The average time interval between cytological and histological diagnosis was six weeks. CIN III nuclei in CS and PEC specimen were Thionin-Feulgen stained and digitized. Beside the visual classification of DNA ploidy patterns also the 2.5c and 5c exceeding rates and the specimen mean and standard deviation values of 21 photometric features were analyzed. It was shown that there was a significant correlation between DNA ploidy patterns in corresponding PEC and CS specimen. The 2.5c and 5c exceeding rates were however significantly higher in the CS specimen. High resolution cytophotometric analysis of cell nuclei in CS and PEC specimen showed significant differences for a large number of nuclear photometric features. These findings can possibly be explained by differences in selection of CIN III cells from CS and PEC specimen and by differences between fixation procedures as used for the two techniques. It was concluded that cytophotometric data of CS and PEC specimen representing CIN III lesions should not be regarded as interchangeable among each other.

INTRODUCTION

In surgical pathology quantitative morphological analysis of nuclei stained with a DNA specific stoichiometric stain may be valuable for diagnostic purposes and in predicting the biological behavior of malignant and premalignant lesions. For an optimal result of cytometrical analysis, accurate tissue-sampling is essential. Many sampling and preparation methods have been described, including several

techniques for enzymatical isolation of cell nuclei from fresh or formalin fixed paraffin-embedded tissue (7,8,13,17,21,27,28). Further techniques were developed for measurements in 4 or 12 micrometer thick paraffin tissue sections, cytological imprints, direct cytological smears, fine needle aspirates and monolayers (3,5,6,10, 13,29). Quantitative analysis of similar lesions using different sampling and preparation techniques may give conflicting results as was shown by an earlier study of 37 breast tumors by Auer et al (3). They demonstrated a weak correlation between DNA ploidy patterns as obtained from three techniques: microspectrophotometric analysis of fine needle aspirates, slide cytophotometric analysis of 4 micrometer tissue sections and flow cytometric analysis of suspensions.
Recently we presented the results of a cytophotometric study, demonstrating that CIN III lesions could be subdivided in two subgroups, possibly indicating progressive and non-progressive lesions (15). These data were established by an analysis of nuclei isolated from paraffin-embedded cervical tissues. It is not known whether these cytophotometric findings can be applied to cytological cervical smears.
In the present study of a group of 22 patients cytophotometric analysis, including DNA ploidy pattern analysis, 2.5c and 5c exceeding rate analysis and high resolution cytophotometric analysis, was performed in routinely prepared cytological smears and in cytospin specimen retrieved from selected areas of paraffin-embedded histological material, both representing CIN III lesions. The aim of the study was to elucidate the consequences of these analysis in CIN III lesions of the same patients using these two preparation techniques.

MATERIAL AND METHODS

Patient group.

A group of 22 women with a cytological and histological diagnosis of Cervical Intraepithelial Neoplasia grade III, was studied. The patients, who were treated for CIN III at the University Hospital Nijmegen, were selected because of a relatively short time interval between the cytological and histological diagnosis. The average interval period was 6 weeks, the range was 0 to 19 weeks.

Digitization.

Nuclei of routinely prepared cytological cervical smears (CS) and paraffin-embedded tissue derived cytospin (PEC) specimen were measured through a 40 x objective using the Nijmegen Image Analysis System (NIAS). The NIAS system is composed of an Orthoplan (Leitz Wetzlar, W.-Germany) scanning microscope, provided with a linear diode array (Fairchild, USA), which was interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Manyard, MA). Thionin-Feulgen stained nuclei were measured with monochromatic light at a wavelength of 585 nm (band width 20 nm). The sampling density was four pixels per micrometer. Nuclei were automatically located in the digital images by grey level boundary following.

Cytophotometric analysis.

Twenty-one nuclear features, describing geometry, density and texture, were extracted from the digitized image (11,16,23,24,25). The nuclear features are listed in table 1. The DNA content of nuclei was expressed as DNA-index (DI), defined as the integrated optical density of a nucleus divided by the median value of integrated optical densities of the internal control nuclei. For each specimen mean and standard deviation of the basic nuclear features were calculated to serve as specimen features.

Cytological material.

The cytological smears were routinely prepared according to the following protocol. Superficial cells were scraped off from the surface of the uterine cervix, using a wooden spatula with a pointed end, immediately put on slides and fixed with a mixture of isopropanol, acetone, poly-oxyethylene and methylene blue (Pro-Fixx, Lerner Laboratories, Pittsburgh, PA, USA). For routine diagnostic purposes the slides were stained according to the Papanicolaou technique, using a CytoTek-TissueTek II slide stainer (Miles Nederland b.v., Weesp, NL). The cytological diagnosis was based on the presence of single and/or clusters of atypical cells that were regarded as diagnostic for CIN III lesions. As a consequence of the sampling technique with a spatula, the majority of diagnostic cells came from the superficial layers of the CIN III epithelium.

Table 1: List of photometrical nuclear features.

TEXTURE	
Runlength	
1:SRL	Short Runlength Emphasis
2:LRL	Long Runlength Emphasis
3:GLD	Greylevel Distribution
4:RLD	Runlength Distribution
5:RPC	Run Percentage
Cooccurrency	
6:HOMOG	Homogeneity
7:CONTR	Contrast
8:INVCON	Inverse Contrast
9:ENTRO	Entropy
10:MXTRP	Maximum Transition Probability
11:DMOM1	First Diagonal Moment
12:DMOM2	Second Diagonal Moment
13:TRISY	Triangular Symmetry
DENSITY	
14:DI	DNA Index
15:ADI	Average DNA Index
16:SDDI	Standard Deviation of DNA Index
17:M3DI	Third Moment of DNA Index
GEOMETRY	
18:AREA	2-D Area of the nucleus
19:CIRC	Circularity
20:GMRAT	Geometrical Moments Ratio
21:MASSEC	Mass Eccentricity; DNA margination

CIN III cells originating from basal and intermediate layers of the epithelium were thought to be rarely present. Normal superficial and some intermediate cells and inflammatory cells were in general also present in these slides.

For cytophotometric analysis the coverslips of the slides were removed using xylol and rehydrated according to the following sequence, ethanol 96% (twice), ethanol 70%, ethanol 40% and distilled water. Thereafter the specimen were fixed in a mixture of methanol, 37% formaldehyde and acetic acid (85-10-5 by volume) for one hour, rehydrated, treated with acid hydrolysis (5 N HCL) at 22^0 C for 60 minutes and stained with the Thionin-Feulgen stain. No cytoplasmic counterstain was performed. An example of a resulting cytological specimen is shown in figure 1.

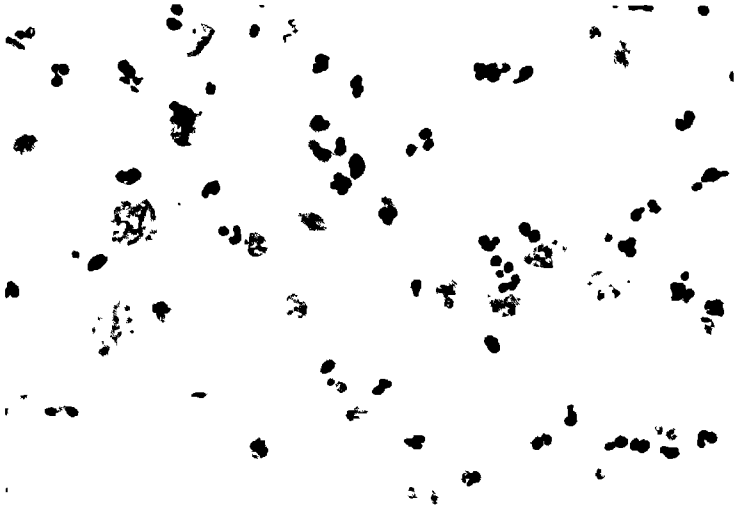

Figure 1: A detail of a cytological smear specimen (CS) representing CIN III cell nuclei (Thionin-Feulgen stain, magnification: 230x).

A mean number of 153 diagnostic CIN III cells (range 54-214) per specimen was selected for measurements. As an internal control sample in each specimen 20 cervical intermediate cells were selected. The median values of the integrated optical densities (MD.IOD) of the 22 samples of intermediate cells ranged from 140.9 to 218.4 (mean: 170.7). The mean value of the coefficients of variation of the samples was 9.5%. Together with the intermediate cells also 20 granulocytes were measured. The MD.IOD values of the 22 samples of granulocytes ranged from 125.5 to 182.5 (mean: 149.7). The mean value of the coefficients of variation of the samples was 8.7%. Regression analysis of MD.IOD values of the intermediate cell nuclei (IODi) and the granulocyte nuclei (IODg) showed the following relation: IODg = 0.87 x IODi.

Paraffin-embedded histological material.

The tissue samples were processed routinely, which included overnight fixation in buffered formalin 4% and embedding in paraffin. Thereafter 50 micrometer tissue sections were cut, deparaffinized in xylene and rehydrated in graded alcohols to distilled water. CIN III areas were selected in the 50 micrometer tissue sections, using a scalpel under a dissecting microscope (14,21). The selected

CIN III areas were then incubated with 0.1% protease of 37^0 C for 60 minutes. Incubation was terminated by adding 4-5 ml cold phosphate buffered saline (PBS), after which the tubes with the CIN III fragments were put into ice. The nuclei were washed twice in PBS and centrifuged. The cell suspension in fetal calf serum was transported on a slide with a cytocentrifuge apparatus (Shandon, U.K., 10 min. x 500 rpm). The slides were briefly air dried and fixed for 1h in a mixture of methanol, 37% formaldehyde and acetic acid (85-10-5 by volume). Nuclei were than treated with acid hydrolysis (5 N HCL) at 22^0 C for 60 minutes and stained with the Thionin-Feulgen stain. The resulting cytospin specimen, of which an example is shown in figure 2, contained cell-nuclei from the superficial, intermediate and basal layers of the CIN III epithelium.

Figure 2: An example of a cytospin specimen (PEC) representing CIN III cell nuclei (Thionin-Feulgen stain, magnification: 230x).

A mean number of 173 nuclei (range: 66-200) were randomly chosen per specimen and used for cytophotometric analysis. As an internal control 20 lymphocytes were selected from the PEC specimen. The median values of the integrated optical densities (MD.IOD) of the 22 samples of the lymphocytes ranged from 76.8 to 186.8 (mean: 117.3). The mean of the coefficients of variation of the samples was 8.1%.

Interpretation of DNA histograms.

The DNA histograms were displayed using a bin-size of 0.1. The DNA ploidy patterns of cytological and histological cytospin specimen were visually classified as DNA-diploid, DNA-polyploid, or DNA-aneuploid, according to the following definitions (2,14,15,18). In a diploid pattern a distinct G0/G1 peak is found in the (near-)diploid (2C; 0.9<DI<1.1) region with a small proportion of cells in S and G2/M (4C) mode, as in normal tissue. This type of DNA ploidy pattern has been called a type I DNA-histogram by Auer et al (2). In polyploidy distinct peaks were present in the diploid and tetraploid (4C; 1.8<DI<2.2) regions, or in the diploid, tetraploid and octaploid (8C; 3.6<DI<4.4) regions. Very few cells or none at all had DNA values corresponding to the DNA synthesis phase of normal cells. This pattern corresponds with the type II DNA-histogram by Auer et at. Ploidy patterns were considered aneuploid in all other cases. These patterns combine the features of the Auer DNA histograms types III and IV.

2.5c and 5c exceeding rate.

An additional description of the DNA distribution in the PEC and CS specimen, was obtained by calculation of the 2.5c and 5c exceeding rates. These were defined as the percentages of cell nuclei having DI values of DI>1.25 and DI>2.5, respectively.

Statistical Analysis.

Differences in the DNA ploidy patterns and in the exceeding rates of CS and PEC specimen were tested using the paired t-test or signed rank test (Wilcoxon), depending of the distribution of the feature values. The correspondence between ploidy patterns was analyzed by rank correlation coefficients. For the high resolution cytophotometric analysis the Pearson correlation coefficients were calculated to test the relationship between the corresponding features in the CS and PEC specimen. This was performed in the complete groups of lesions and in two subgroups of nuclei. These subgroups (containing at least 15 nuclei per subgroup) were: a) (near-)diploid nuclei with DI values between 0.8 and 1.2, and b) (near-)tetraploid nuclei with DI values between 1.6 and 2.4. These subgroups were chosen assuming that they contained comparable subpopulations for both prepara-

tion techniques. The influence of a shift towards different ploidy-levels that might appear as a consequence of differences between both techniques, could as such be reduced.

RESULTS

DNA ploidy analysis.

Table 2 shows the results of the DNA ploidy analysis in PEC and CS specimen of CIN III lesions.

Table 2: Results of DNA ploidy analysis of corresponding cytological smears (CS) and cytospin specimen derived from paraffin-embedded tissues (PEC), both representing CIN III lesions.

	PEC			
	Diploid	Polyploid	Aneuploid	Total
CS				
Diploid	0	0	0	0
Polyploid	3	5	0	8
Aneuploid	1	4	9	14
Total	4	9	9	22

In the 22 PEC specimen four (18%) cases were classified as diploid; nine (41%) cases were polyploid and nine (41%) cases were aneuploid. In the corresponding 22 CS specimen no diploidy occurred. Eight (36%) specimen showed polyploidy and fourteen (64%) were aneuploid. The visual classification of the ploidy patterns of the corresponding PEC and CS specimen were in agreement in fourteen cases. There was a significant ($p<0.001$) correlation of 0.62 between the corresponding ploidy patterns obtained by the two sampling techniques.
Figures 3 and 4 give examples of comparable polyploid and aneuploid DNA patterns in corresponding PEC and CS specimen.
In eight cases there was no agreement between the corresponding ploidy patterns. The four CIN III lesions that were classified as diploid in the PEC specimen, showed a polyploid pattern in three cases of the CS specimen and a aneuploid pattern in one case.

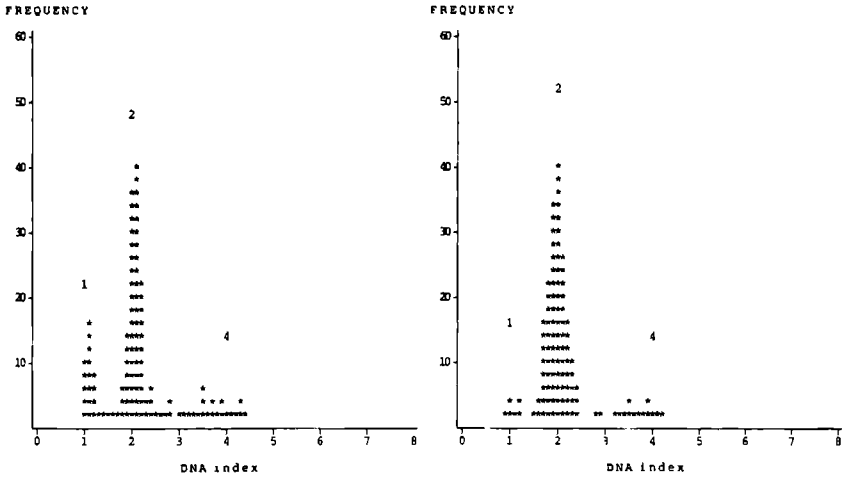

Figure 3 Polyploid DNA pattern of a CIN III lesion in corresponding PEC (left histogram) and CS (right histogram) specimen

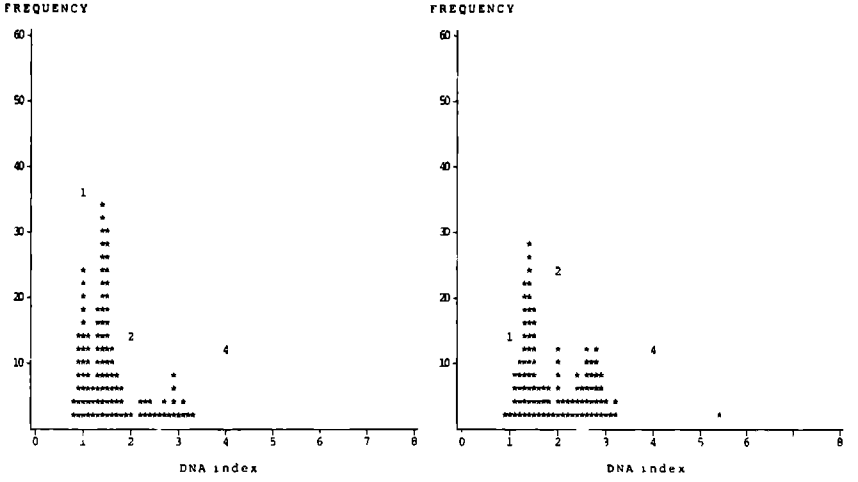

Figure 4 Aneuploid DNA pattern of a CIN III lesion in corresponding PEC (left histogram) and CS (right histogram) specimen

In figure 5A and 5B the results of a case are presented that showed diploidy in the PEC specimen and a polyploid pattern in the CS specimen. An explanation for these findings might be that in the CS specimen exclusively the diagnostic cells were selected and measured, while in the PEC specimen cells were randomly selected and measured. This links up with the finding that the DNA ploidy pattern changed towards polyploidy/aneuploidy, if in the diploid PEC specimen exclusively the severe atypical ("diagnostic") cell nuclei were selected and remeasured, as is illustrated in figure 5C.

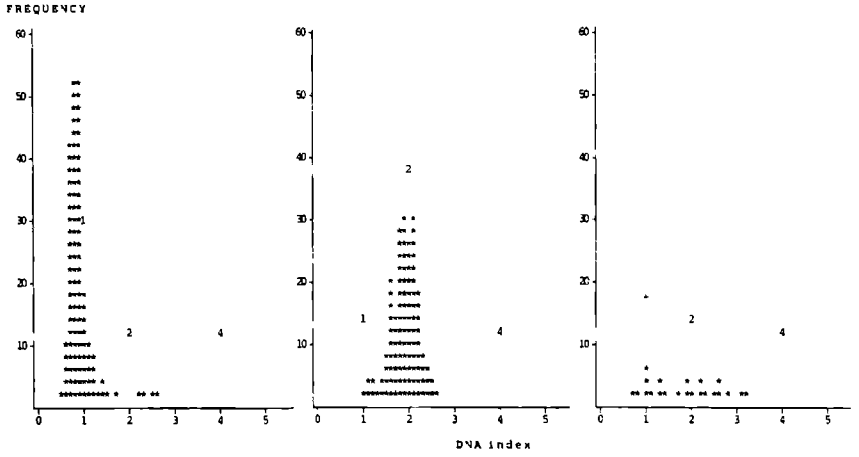

Figure 5: A: Diploid DNA pattern of a CIN III lesion in the PEC (left histogram) specimen.
B: Polyploid DNA pattern of a CIN III lesion in the corresponding CS (middle histogram) specimen.
C: Scattered DNA ploidy values of the PEC (right histogram) specimen, after selective measuring.

Five of the nine cases of polyploidy as measured in the PEC specimen, were also classified as polyploid in the CS specimen. Four were classified as aneuploid. In these latter four cases, the change of ploidy pattern was initiated by the presence of a clear aneuploid fraction of cells in the 6-7c (DI=3-3.5) area, as is illustrated in figure 6. All PEC specimen that were classified as aneuploid, demonstrated also aneuploidy in the cytological smears. In general there was a significant ($p<0.005$) shift from diploid patterns towards polyploidy and aneuploidy and from polyploidy towards aneuploidy, in PEC specimen and CS specimen respectively.

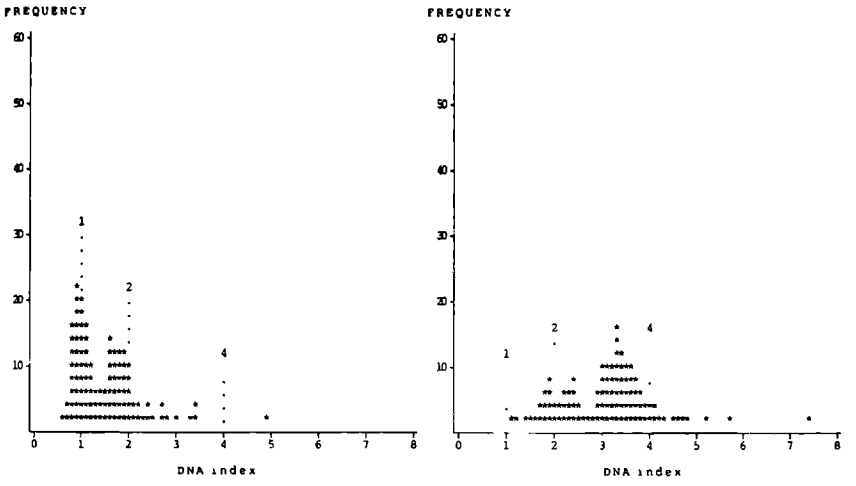

Figure 6: DNA ploidy patterns of a CIN III lesion in corresponding PEC and CS specimen: polyploid pattern in PEC (left histogram) and aneuploid pattern in CS specimen (right histogram).

The 2.5c and 5c exceeding rate.

Figure 7 demonstrates the results of the 2.5c (DI = 1.25) and 5c (DI = 2.5) exceeding rate analysis of the 22 PEC and CS specimen.

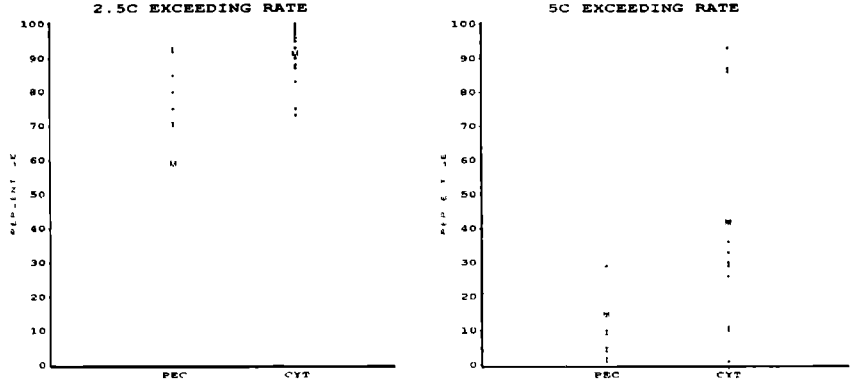

Figure 7: Distribution of the 2.5c and 5c exceeding rate of 22 cases of corresponding PEC and CS specimen, both representing CIN III lesions.

The mean percentage of nuclei, that had DNA values exceeding 2.5c in the PEC specimen, was 59%, and ranged between 5% and 93%. In cytological smears the mean percentage of the 2.5c exceeding rate was 91%, and the range was 54%-100%. The mean percentage of PEC nuclei exceeding 5c was 14%, ranging between 1% and 48%. In the cytological smears this percentage was 42%, ranging from 1% to 93%. The 2.5c and 5c exceeding rate of the CS specimen were significantly higher then those of the PEC specimen ($p<0.0001$). For 19 cases the 2.5c exceeding rate was highest in CS specimen; they were identical in CS and PEC in two cases and highest in PEC specimen in one case. For the 5c exceeding rate these figures were: 19, one and two cases respectively.

High resolution cytophotometric analysis.

The Pearson correlation procedure, using the specimen mean and standard deviation values of 21 photometric features, was performed in the complete group of 22 cases, in a subgroup of cases with cell nuclei, that were in the (near)-tetraploid DI value area and in a subgroup of cases with cell nuclei, that were in the (near-)diploid DI value area. The results of the analysis in the complete group of specimen indicated that there was a significant correlation between PEC and CS specimen for just three features: the mean and standard deviation of AREA and the mean of DNA-INDEX ($p<0.05$). In the subgroup of (near-)tetraploid cell nuclei no correlation between the corresponding specimen feature values was found. The correlation analysis that was performed in the (near-)diploid subpopulation indicated that three specimen mean feature values (LRL, RPC and INVCON) correlated in the PEC and CS specimen. However only 4 of the 22 specimen had sufficient ($N>=15$) (near-)diploid cell nuclei in both the PEC and CS specimen. To analyze the differences between the feature values of the (near)-tetraploid subgroups of the PEC and CS specimen a paired t-test or signed rank test was performed. The results showed significant differences ($p<0.005$) between the feature values of PEC and CS specimen concerning the specimen mean and standard deviation values of six features (LRL, RPC, CONTR, DMOM2, CIRC and GMRAT), and the specimen mean values of another eight features (SRL, GLD, HOMOG, INVCON, ENTRO, DMOM1, TRISY and MASSEC) (Table 3). The highest values for the mean and standard deviation of RPC, CONTR and DMOM2 and for the mean values of SRL, ENTRO, DMOM1, TRISY, CIRC and MASSEC were found in the CS specimen. The highest values for the mean and

standard deviation of LRL and GMRAT, for the mean values of HOMOG and INVCON and the standard deviation of CIRC were found in the PEC specimen.

*Table 3: Mean (M) and standard deviation (S) values of nuclear photometric features of (near)-tetraploid (1.6<DI<2.4) subgroups of nuclei from PEC and CS specimen, both representing CIN III lesions (N = 19; highest values of significantly different features are underlined).**

	PEC		CS	
	MEAN	ST.DEV.	MEAN	ST.DEV.
M.SRL	0.27	0.06	0.37	0.04
M.LRL	107	39	59	19
M.GLD	0.19	0.04	0.15	0.01
M.RLD	0.15	0.03	0.16	0.01
M.RPC	0.15	0.03	0.21	0.03
M.HOMOG	0.046	0.005	0.039	0.003
M.CONTR	0.98	0.24	1.46	0.23
M.INVCON	0.71	0.04	0.66	0.02
M.ENTRO	1.43	0.05	1.50	0.03
M.MXTRP	0.098	0.004	0.092	0.003
M.DMOM1	3.19	0.44	3.93	0.34
M.DMOM2	0.32	0.05	0.41	0.04
M.TRISY	0.09	0.02	0.12	0.02
M.DI	1.98	0.12	2.05	0.08
M.ADI	0.0010	0.0003	0.0013	0.0003
M.SDDI	0.0004	0.0002	0.0005	0.0001
M.M3DI	0.0008	0.0003	0.0009	0.0002
M.AREA	2280	560	1795	565
M.CIRC	0.76	0.03	0.81	0.02
M.GMRAT	2.7	0.5	1.9	0.2
M.MASSEC	0.45	0.01	0.47	0.01
S.SRL	0.083	0.015	0.070	0.017
S.LRL	48	13	31	11
S.GLD	0.06	0.04	0.03	0.02
S.RLD	0.06	0.03	0.04	0.01
S.RPC	0.04	0.01	0.052	0.006
S.HOMOG	0.0056	0.0009	0.0053	0.0012
S.CONTR	0.30	0.08	0.45	0.05
S.INVCON	0.043	0.007	0.047	0.007
S.ENTRO	0.054	0.007	0.058	0.009
S.MXTRP	0.008	0.002	0.008	0.001
S.DMOM1	0.54	0.09	0.63	0.08
S.DMOM2	0.059	0.011	0.074	0.009
S.TRISY	0.042	0.019	0.045	0.011
S.DI	0.17	0.04	0.20	0.03
S.ADI	0.00027	0.00012	0.00040	0.00015
S.SDDI	0.00014	0.00006	0.00015	0.00005
S.M3DI	0.00025	0.00010	0.00028	0.00009
S.AREA	625	175	565	185
S.CIRC	0.059	0.008	0.048	0.010
S.GMRAT	1.14	0.26	0.71	0.21
S.MASSEC	0.019	0.004	0.022	0.002

* Significance level of the paired t-test or signed rank test (Bonferroni correction): $\alpha = 0.0012$.

The morphologic translation of these cytophotometric findings was that CIN III nuclei in CS specimen appear to have on the average a more finely (and less coarse) granular chromatin, a higher contrast between the chromatin particles, more DNA margination, a more rounded shape and less variation in shape. Furthermore the nuclei in the CS specimen appear to have more variation in graininess and in contrast between the chromatin particles.

DISCUSSION

The aim of the present study was to compare DNA-measurements of corresponding CIN III lesions in cytological smears and cytospin specimen, derived from paraffin-embedded tissue. The results of DNA ploidy analysis showed that there was a significant correlation between ploidy patterns of the two types of sampling-/preparation techniques. There was however a significant shift from diploidy towards polyploidy/aneuploidy and from polyploidy towards aneuploidy in PEC and CS specimen respectively. This finding was supported by the results of the analysis of the 2.5c and 5c exceeding rate, that indicated a significantly higher percentage of cells with a higher DNA content in CS specimen.
In respect to these differences between corresponding PEC and CS specimen the following considerations were made.

1) The problem of sample representativety and cell selection. In PEC specimen the complete region of the CIN III lesion was selected in the thick paraffin-embedded tissue sections, and as such cells from the intermediate and basal layers of the epithelium were also thought to be present. The cell nuclei were therefore randomly measured. Despite this selection a small number of normal cervical epithelial and stromal cells may still have been present in PEC specimen, thus influencing the outcome of the measurements. Cervical smears were taken with a wooden spatula and were thought to render mostly superficial cells, with a large admixture of cervical normal epithelial and inflammatory cells. The selection and measurement of CIN III cells in CS specimen was based on the presence of diagnostic CIN III cell nuclei, according to criteria regarding to size, shape and chromatin pattern of the nuclei (22). Random selection and measurement of nuclei in CS specimen is not appropriate because of the varying admixture with inflammatory and epithelial cells. If the cell nuclei in the PEC specimen, were selected according to the same diagnostic criteria as used for the CS specimen, the results

of ploidy analysis of the PEC specimen changed towards the pattern as found for the CS specimen. These findings could indicate that in CS specimen a "superselection" of CIN III nuclei with a higher DNA content was carried out and that the lower-ploid cell nuclei were discarded. It is possible that these lower-ploid cell nuclei do contribute to the potential progressive behavior of the CIN III lesion. In this respect it is of interest that earlier studies have shown that patients with cervical squamous cell carcinoma with a low ploidy level seemed to have a less favorable prognosis than patients with a more aneuploid DNA pattern (1,19).

2) Another reason for the dissimilarity between the results of cytometrical analysis of CS and PEC specimen can be the different processing of the specimen. In this respect pitfalls concerning fixation, staining, functional differences between cells and instrumentation have been indicated by studies by Auer et al, Schulte et al, Mayall and Giroud et al (3,12,20,26). In the present study the staining procedures and the measurement instrumentation were similar for the CS and PEC specimen. The cell samples were from the same patients and arguably from the same lesions. Therefore it seems likely that, beside the cell selection bias as mentioned above, our present findings may be explained by differences in the fixation steps of the CS and PEC specimen. In this respect it is of interest to realize that acid hydrolysis as used in the Feulgen reaction gives a significant loss of protein in ethanol-acetone-fixed cells as compared to formalin-fixed cells (3,12,26). These factors may influence the chromatin structure and in turn influence the DNA stainability. Our present findings of low correlation for cytophotometric analyzed nuclear chromatin features in the complete groups of PEC and CS specimen and for the subpopulations of (near-)tetraploid cells add to these earlier findings. The morphologic translation of the differences in the (near-)tetraploid cell nuclei in the PEC and CS specimen are in agreement with earlier observations, that formalin fixed cell nuclei tend to have a more coarse chromatin pattern than alcohol-based fixatives. The mean AREA value in PEC specimen was higher, although not statistically significant, than in CS specimen. This adds to earlier findings of Baak et al and Fox who noted that formaldehyde fixation gave little shrinkage of tissues and nuclear area (4,9). Another variable that may influence the outcome of the comparison of CS and PEC specimen is the enzymatic isolation procedure as used for the paraffin-embedded tissues. A selective loss of nuclear DNA or of entire nuclei may take place during enzymatic treatment (3).
In summary the results of the present cytophotometric study of corresponding CS and PEC specimen, both representing CIN III lesions, indicate that DNA

ploidy patterns of corresponding CIN III lesions show a significant correlation. The 2.5c and 5c exceeding rates of CS specimen are however significantly higher in the vast majority of cases. The high resolution cytophotometric analysis of a subgroup of (near-)tetraploid cell nuclei in CS and PEC specimen showed significant differences for the majority of nuclear photometric features. These differences could possibly be explained by the variance in selection of CIN III cells, and by the differences between the fixation steps as used in the CS and PEC procedures. As a consequence it is concluded that cytophotometric data of CS and PEC specimen representing CIN III lesions should not be regarded as interchangeable among each other.

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CHAPTER 7

PROGRESSIVE CERVICAL INTRAEPITHELIAL NEOPLASIA: A CYTOPHOTOMETRIC ANALYSIS

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Abstract DNA ploidy analysis, 2.5C and 5C exceeding rates and high resolution cytophotometric analysis were performed in 73 cervical cytological smears with cytologial diagnosis of CIN I, II, III and invasive carcinoma from 57 women, to identify prognostic parameters. From four cervical screening registries a group of 28 women was selected on basis of a final histologic diagnosis of cervical invasive squamous cell carcinoma, with previous cervical smears consistent with CIN I, II and III lesions and who were left untreated. A second group of 29 patients, who were treated for cytologically diagnosed CIN III was regarded to represent a mixture of progressive, persistent and regressive lesions. In 64% of cervical smears from the group of patients who had developed invasive cancer, a DNA-aneuploid pattern was found both in CIN and in invasive lesions. DNA polyploidy was present in 36% of both of these lesions. It was concluded that DNA polyploid CIN lesions should not be regarded as possibly reactive or regressive. DNA diploidy did not occur in these CIN or invasive cancer cases. The 2.5C exceeding rate increased with increasing severity of the CIN lesion. A high resolution cytophotometric analysis demonstrated a trend-like pattern of a number of feature values in lesions with increasing severity, thus from CIN I and CIN II to CIN III and invasive cancer. In a cluster analysis based on the results of high resolution cytophotometric analysis, a subdivision of CIN III lesions with unknown malignant potential was possible into a group of lesions with similar feature values as a group of progressive CIN III and a second group of lesions that had feature values that were dissimilar to progressive CIN III. It was suggested to apply a classifier based on a combination of age, high resolution cytophotometric data and ploidy patterns in a prospective study of cytologically detected CIN-lesions.

The natural history of premalignant cervical lesions has not been solved. There are considerable differences in the results of follow-up studies, depending on issues like different follow-up period, the taking of biopsies during follow-up period, different interpretation of cytologic and histologic aspect, and different statistical approaches (4,8,9,13,15,25,27-29,31,35,36,38,39,43). In an overview article, Koss recently stated that at the most one in ten cervical precancerous lesions is likely to progress to invasive cancer, when left untreated (26). It is however not possible to predict by cytopathologic, histopathologic or colposcopic examination which cervical intraepithelial neoplasia (CIN) will finally become invasive. As a consequence all cases of CIN are being treated as premalignant, potentially progressive lesions.

As a possible tool to predict the malignant potential of CIN, cytogenetic and cytometric DNA studies have been considered. Cytogenetic studies in cervical biopsy specimen have indicated that aneuploidy is frequent in CIN II and III lesions, although (near-)diploidy does occur (1,2,24,41). It was mentioned that carcinoma in situ may have undergone less chromosomal loss and fewer structural changes than invasive cervical carcinoma, that often appeared to be near-triploid (2). Spriggs and coworkers in a study of 28 cases of CIN and microinvasive carcinoma, concluded that no chromosomal features were distinctive of dysplasia, carcinoma in situ, microinvasive or invasive carcinoma (41). Microspectrophotometric, flow and image cytometric DNA ploidy measurements have increasingly been performed in cervical scrapings, in fresh biopsy material and in paraffin sections (5-7,10,11,14,16,17,23,30,32,40,45). Some investigators have indicated that CIN with an aneuploid DNA pattern has a higher rate of persistence or recurrence than lesions with a diploid or polyploid pattern (6,11,32). In other studies however no significant differences could be demonstrated between CIN II lesions that progressed to carcinoma in situ and those that regressed to normality (30). In a study of DNA-measurements in nuclei, isolated from paraffin-embedded CIN III and CIN III-like lesions adjacent to invasive squamous cell cancer (CIN-INV), we showed that DNA-diploidy did occur in CIN lesions, especially in a group of younger patients with CIN III-like lesions adjacent to invasieve cancer (16). This finding correlated with data that showed that about half of the cases of cervical invasive squamous cell carcinoma were (near-)diploid (2,17).

A recent high resolution cytophotometric analysis of nuclei retrieved from paraffin-

embedded CIN III and CIN.INV lesions, using texture, density and geometry features, indicated that in the group of CIN III lesions a subgroup of lesions was present with similar photometric feature values as in the vast majority of CIN.INV lesions, indicating a possible progressive potential of these CIN III lesions. These results showed that high resolution cytophotometric analysis may be of help in the distinction between progressive and non-progressive cervical intraepithelial lesions (18).

For the present study, 73 cervical cytologic smears were selected. 44 were cervical smears from patients who, after an initial cervical smear after a variable interval and without intermittend treatment, developed cervical invasive squamous cell carcinoma (INV). These cervical smears were used in DNA-ploidy analysis and a high resolution cytophotometric analysis. The aim of the study was to describe DNA ploidy patterns, 2.5c and 5c exceeding rates and high resolution cytophotometric feature values in progressive CIN I, II, III and INV lesions. Furthermore it was aimed to test previous findings of features indicating a possible progressive potential on CIN III lesions with a known progressive course and to analyze whether these features would identify subgroups in CIN III lesions with an unknown biological behavior because of intercurrent treatment.

MATERIAL AND METHODS

Patient group.

From four cervical screening registries a group of 28 women was selected on the basis of a final histologic diagnosis of cervical invasive squamous cell carcinoma, having had previous cervical smears (CS), consistent with CIN I, II or III lesions (table 1) and who were left untreated. The original cytologic cervical smears had been misinterpreted as negative or showing only minor abnormalities or the patients had been lost to follow-up. The histologic diagnosis was microinvasive carcinoma (stage 1A) in five cases and macroinvasive carcinoma (stage 1B or higher) in 23 cases. The mean time interval between the cytologic and histologic diagnosis was 30 months for the microinvasive carcinomas and 33 months for the macroinvasive carcinomas. The CIN lesions of these 28 patients are labelled with the prefix "p" for their progressive course (pCIN I-III).

Table 1: Distribution of cervical smears, subdivided for the 57 patients according to the different combinations of cytologic diagnosis.

	No. of patients	p CIN I	p CIN II	p CIN III	INV	No. of smears
	1	1		1	1	3
	1	1	1			2
	2	2		2		4
	3		3	3		6
	8			8	8	16
	1	1				1
	1		1			1
	9			9		9
	2				2*	2
Total (progr)	28	5	5	23	11	44
				u CIN II		
Total (unknown)	29			29		29
Total	57	5	5	52	11	73

* The corresponding previous CIN lesions contained less than 20 measurable cells and were discarded from the study.

A second group of 29 patients was selected, who were treated for cytologically diagnosed CIN III at the University Hospital Nijmegen. This group of CIN III lesions was regarded to represent a mixture of progressive, persistent and regressive lesions (18). The CIN III lesions of these patients are further indicated by the prefix "u" for unknown progressive potential (uCIN III).

Cytologic material.

Cytologic smears had been routinely prepared by scraping the surface cervical mucosa with a wooden spatula. The material was then put on slides and fixed. For routine diagnostic purposes the slides were stained according to the Papanicolaou technique. The cytologic diagnosis made at rescreening, was based on the presence of singly lying or clusters of atypical cells that were regarded as diagnostic for CIN I, II, III or INV lesions. Usually normal superficial and intermediate

epithelial cells and inflammatory cells were also present in these slides. For cytophotometric analysis the coverslips of the slides were removed using xylol and the specimens were then rehydrated according to the following sequence, ethanol 96% (twice), ethanol 70%, ethanol 40% and distilled water. Thereafter specimens were fixed in a mixture of methanol, 37% formaldehyde and acetic acid (85-10-5 by volume) for one hour, rehydrated, treated with acid hydrolysis (5 N HCL) at 22^0 C for 60 minutes and stained with the Pararosanilin (Schiff)-Feulgen stain. No cytoplasmic counterstain was used. A mean number of 129 (range 23-200) diagnostic cells per specimen was selected for measurements. As an internal control from each specimen 20 cervical intermediate squamous cells were measured. The median values of integrated optical densities (MD.IOD) of intermediate cells, present in the 73 samples, ranged from 78 to 268 (mean: 196). The mean value of the coefficients of variation of the controls was 11,7%.

Digitization.

Nuclei of the cytologic smears were measured with the Nijmegen Image Analysis System (NIAS). The NIAS system is composed of an Orthoplan (Leitz Wetzlar, W.-Germany) scanning microscope, equipped with a linear diode array (Fairchild, USA), which was interfaced to a MINC minicomputer with a LSI 11/23 microprocessor (Digital Equipment Corp., Manyard, MA). Pararosanilin (Schiff)-Feulgen stained nuclei were measured with monochromatic light at a wavelength of 565 nm (band width 20 nm), using a 40x objective. The sampling density was four pixels per micrometer. Nuclei were automatically located in the digital images by grey level boundary following.
Twenty-one nuclear features, describing geometry, density and texture were extracted from the digitized nuclear images (12,20,33,34,37). The DNA content of nuclei was expressed as DNA-index (DI), defined as the integrated optical density of a nucleus divided by the median value of integrated optical densities of internal control nuclei. For each specimen mean and standard deviation of all nuclear features were calculated and used as "specimen features".

Interpretation of DNA histograms.

DNA histograms were displayed using a bin-size of 0.1. DNA ploidy patterns were visually classified as DNA-diploid, DNA-polyploid, or DNA-aneuploid, according

to the following definitions (3,17,21,22). In a DNA-diploid pattern a distinct G0/G1 peak is found in the (near-)diploid (2C; 0.9<DI<1.1) region with a small proportion of cells in S and G2/M (4C) mode, as in normal tissue. In DNA-polyploidy distinct peaks were present in the diploid and tetraploid (4C; 1.8<DI<2.2) regions, or in the diploid, tetraploid and octaploid (8C; 3.6<DI<4.4) regions. Very few cells or none at all had intermediate DI values. Ploidy patterns were considered DNA-aneuploid in all cases with scattered DI distributions, or uni-, bi-, or multimodal DI distributions, that were non-diploid and non-polyploid.

2.5c and 5c exceeding rates.

The 2.5c and 5c exceeding rates (ER) were defined as the percentages of cell nuclei per specimen, with DI values of DI > 1.25 and DI > 2.5, respectively.

Statistical Analysis.

The first aim of the study was to describe DNA ploidy patterns, 2.5c and 5c exceeding rates and cytophotometric feature values in progressive CIN I, II, III and in INV lesions. Non-parametric tests were used to find differences in exceeding rates and cytophotometric feature values. A trend in feature values with increasing severity of the diagnosis, was tested using the Terpstra test.
The second aim of the study was to determine if the group of uCIN III lesions could be subdivided into two subgroups: 1) a group of lesions with similar feature values to the group of pCIN III lesions and as such possibly representing potentially progressive lesions, 2) a group of lesions with feature values dissimilar to the pCIN III lesions. A stepwise linear discriminant analysis was performed, to select features that could discriminate between uCIN III and pCIN III lesions, assuming these to be good candidates for discrimination of the two subgroups. The stepwise discriminant analysis was based on all 42 mean (M) and standard deviation (S) feature values (after an adequate transformation to "normality") of individual patients. Ward's cluster analysis was carried out using the best discriminating M and S features from the stepwise discriminant procedures and using a set of features as used in a previous study of CIN III lesions (18,42,44). The Ward procedure implies the use of standard deviation-scores of the features. The clustering procedure aimed to reach a division of the uCIN III group under the

condition that the pCIN III group was not further subdivided. In this manner uCIN III lesions could be identified, that had corresponding feature values to the pCIN III group.

RESULTS

Description of pCIN I, II, III and INV.

DNA ploidy analysis.

The results of DNA ploidy analysis in cytologic smears from 33 progressive CIN (5 pCIN I, 5 pCIN II and 23 pCIN III) and from 11 INV lesions are shown in table 2. In these pCIN and INV lesions DNA-diploidy did not occur. Four of the five pCIN I specimen were classified as DNA-polyploid and one as DNA-aneuploid. Two of the five pCIN II specimen showed a polyploid and three an aneuploid DNA pattern. Six (26 %) of 23 pCIN III lesions were DNA-polyploid and 17 (74 %) were DNA-aneuploid. This showed a slight predominance of DNA-polyploidy in pCIN I and of DNA-aneuploidy in pCIN II and pCIN III. In the complete group of pCIN I-III lesions DNA-aneuploidy occurred in 21 cases (64 %), and DNA-polyploidy in 12 cases (36 %). The same ratio applied for the cytologic specimen from invasive carcinomas: four (36 %) cases were DNA-polyploid, seven (64 %) were DNA-aneuploid.

Two or more successive smears could be analyzed in 15 patients with progressive lesions (table 1 and 3): in eight patients with successive pCIN III and INV specimen, in three patients with pCIN II and pCIN III specimen, in two patients with pCIN I and pCIN III specimen, and in one patient with pCIN I and pCIN II. In one patient a pCIN I, pCIN III and an INV specimen were measured.

The classification of the DNA-ploidy patterns of the successive specimen were in agreement in 11 (73%) cases, including the case with three successive smears.

Figure 1 shows examples of DNA-ploidy patterns in three patients with successive CIN and INV lesions. In figure 1A the polyploid DNA patterns of pCIN III and INV lesions of patient no. 3 are shown. Figure 1B demonstrates a DNA-aneuploid pattern of pCIN III and a DNA-polyploid pattern of INV of patient no. 7. Figure 1C presents DNA-aneuploid patterns of patient no. 9 with successive pCIN I, pCIN III and INV lesions. This figure further illustrates that DNA-aneuploid patterns in successive smears are not necessarily identical.

*Table 2: Results of DNA ploidy analysis of all cytologic smears, representing pCIN I, pCIN II, pCIN III, INV and uCIN III lesions.**

	Diploid	Polyploid	Aneuploid	Total
pCIN I	-	4 (80%)	1 (20%)	5
pCIN II	-	2 (40%)	3 (60%)	5
pCIN III	-	6 (26%)	17 (74%)	23
Tot.pCIN I-III	-	12 (36%)	21 (64%)	33
INV	-	4 (36%)	7 (64%)	11
uCIN III	1 (3%)	15 (52%)	13 (45%)	29
Total	1	34	38	73

* pCIN I-III = CIN I-III lesions of patients who have developed an invasive squamous cell carcinoma. uCIN III = CIN III lesions with unknown progressive potential.

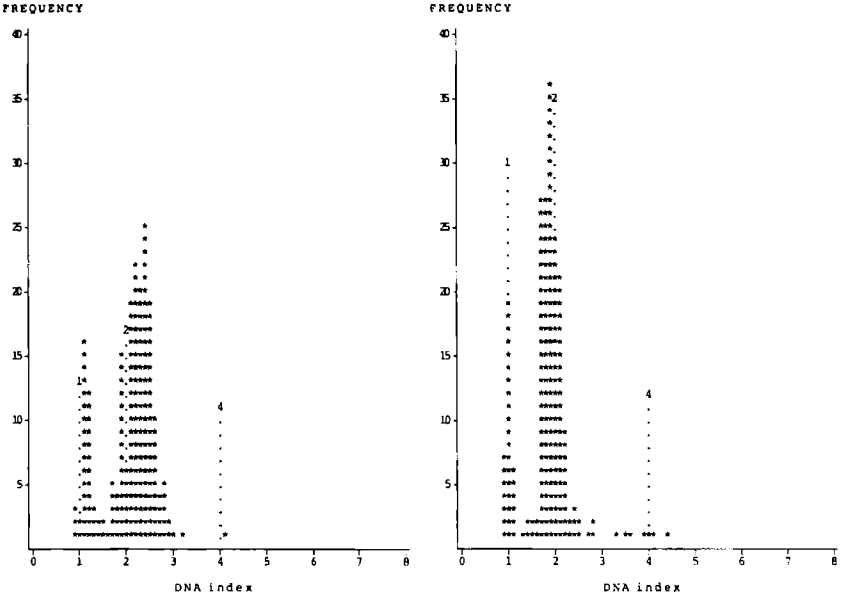

Figure 1A: DNA-polyploid patterns of CIN III (left histogram) and INV (right histogram) lesions of one patient (patient no. 3).

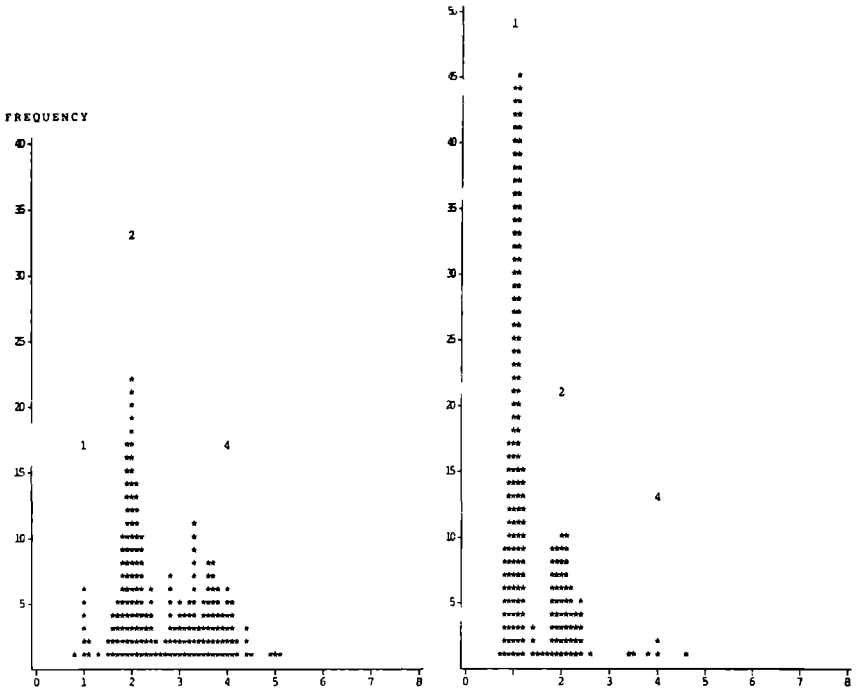

Figure 1B· Aneuploid DNA-patterns of CIN III (left histogram) and a polyploid DNA-pattern of INV (right histogram) lesion of one patient (patient no 7).

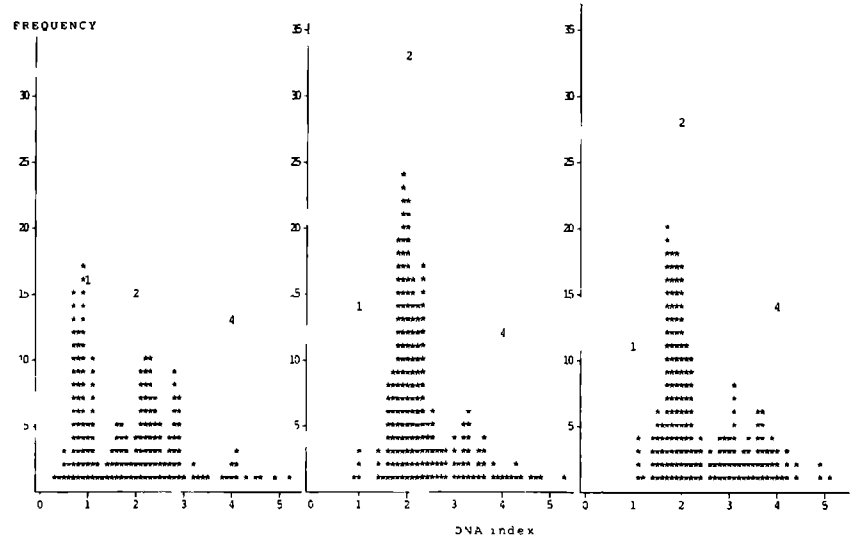

Figure 1C: Aneuploid DNA-patterns of the successive CIN I (left histogram), CIN III (middle histogram) and INV (right histogram) lesion of one patient (patient no. 9) In the CIN I lesion three stemlines seem to be present with near-diploid peaks at DI = 0 7, 0 9 and 1.1. In the near-tetraploid region also three peaks with DI values of 1.6, 2.2 and 2 8. In the near-octaploid region two smaller peaks with DI values of 3.2 and 4.1. In the CIN III and INV lesions this pattern is not as obvious, with, for CIN III, peaks mainly in the near-tetraploid region at DI = 1.9, 2 3 and perhaps at 2.5, and with three small adjacent peaks at DI = 3 0, 3 3, and 3 6. In the INV lesion the main peaks are at DI = 1.7 (possibly also at 1.5 and 2.4), 3.1 and 3.6.

Table 3 Age, cytologic and histologic diagnosis ploidy pattern, 2 5c and 5c ER in cytologic smears, representing successive progressive cervical intraepithelial neoplasia grade I-III (pCIN I-III) and invasive squamous cell carcinoma (INV) of 15 patients

Patient No.	Age	Year of diagnosis	Cytologic diagnosis	Histologic diagnosis	Ploidy pattern	2.5c ER	5c ER
3	58	1974	CIN III		Polyploid	84	15
		1978	INV		Polyploid	84	6
		1978		INV			
4	47	1977	CIN III		Aneuploid	87	26
		1980	INV		Aneuploid	100	62
		1980		INV			
5	35	1976	CIN III		Polyploid	90	6
		1980	INV		Polyploid	94	2
		1980		INV			
6	32	1978	CIN III		Polyploid	42	15
		1981	INV		Polyploid	88	11
		1981		INV			
7	49	1978	CIN III		Aneuploid	95	47
		1981	INV		Polyploid	33	4
		1981		INV			
8	32	1977	CIN II		Aneuploid	83	7
		1980	CIN III		Aneuploid	98	63
		1981		INV			
9	35	1977	CIN I		Aneuploid	61	24
		1981	CIN III		Aneuploid	98	26
		1982	INV		Aneuploid	97	37
		1982		INV			
10	37	1975	CIN II		Aneuploid	100	76
		1976	CIN III		Aneuploid	100	69
		1981		INV			
12	37	1979	CIN III		Aneuploid	99	51
		1983	INV		Aneuploid	81	25
		1983		INV			
14	43	1981	CIN III		Polyploid	80	11
		1983	INV		Aneuploid	97	20
		1983		INV			
15	27	1980	CIN I		Polyploid	53	0
		1983	CIN III		Aneuploid	100	53
		1983		INV			
17	60	1976	CIN I		Polyploid	89	26
		1977	CIN III		Polyploid	95	11
		1977		INV			
18	42	1973	CIN II		Aneuploid	88	19
		1978	CIN III		Aneuploid	90	27
		1980		INV			
26	39	1976	CIN I		Aneuploid	91	13
		1981	CIN II		Polyploid	40	1
		1981		INV			
27	47	1980	CIN III		Aneuploid	88	20
		1983	INV		Aneuploid	96	49
		1983		INV			

Exceeding rates.

The mean and standard deviation values of the 2.5c and 5c exceeding rates by diagnosis are illustrated in table 4. Nine patients with both CIN III and INV diagnosis were selected to test differences in exceeding rates between both diagnosis, using the (paired) Wilcoxon test. No differences were found. In order to avoid statistical dependencies, due to incidental repeated measurements within the same patients, differences between the combined CIN I+II group and CIN III, were tested in two ways, using the Wilcoxon test on six pairs of smears (see table 1) and the Mann-Whitney test for nine smears (CIN I+II) against 17 smears (CIN III). The difference in 2.5c ER for CIN I+II against CIN III was of a borderline significance in both tests ($p=0.07$ and $p=0.06$ respectively). For 5c ER no significant differences were found

Table 4 Mean and standard deviation (between brackets) of the 2.5c and 5c exceeding rates (ER) by diagnosis

	pCIN I	pCIN II	pCIN III	INV
2.5c ER	66 (24%)	70 (29%)	89 (13%)	88 (19%)
5c ER	13 (13%)	22 (31%)	31 (22%)	29 (22%)

High resolution cytophotometric analysis.

Nine patients with both pCIN III and INV diagnosis were selected to test differences in feature values, using the (paired) Wilcoxon test. No differences were found.

As applied for the ER values (see above), differences between the combined pCIN I+II group and pCIN III, were tested in two ways, using the Wilcoxon test on six pairs of smears (see table 1) and the Mann-Whitney test for nine smears (pCIN I+II) against 17 smears (pCIN III). For both tests the same features showed significant differences (M-massec, S-rpc, S-contr, S-invcon, S-entro, S-dmom1 and S-dmom2, Wilcoxon test $p<0.03$, Mann-Whitney test $p<0.01$). A subdivision of pCIN III lesions into lesions with a short or long (> 24 months) time interval prior to the histologic diagnosis of cancer did not influence this result.

To study a trend in the cytophotometric feature values with increasing severity, the Terpstra trend test was used on five pCIN I, five pCIN II and 17 pCIN III independent smears, all showing a decreasing value with severity. In table 5 the

mean and standard deviation values of these features in the groups of pCIN I, pCIN II and pCIN III are given. A translation of these findings into light microscopical cytologic characteristics is that with increasing severity of the progressive CIN, nuclei tend to have less finely granular chromatin, less contrast between the chromatin particles (equivalent to more overall internal contrast), less variation in contrast between the chromatin particles, and less DNA margination.

Table 5 The 11 features having significant decreasing mean (M) and standard deviation (S) values of nuclear photometric features of pCIN I, pCIN II and pCIN III independent lesions

	pCIN I N=5		pCIN II N=5		pCIN III N=17		p*
	M	S	M	S	M	S	
M-srl	0 357	0 033	0 344	0 028	0 327	0 030	0 03
M-rpc	0 200	0 023	0 179	0 027	0 172	0 020	0 03
M-contr	1 41	0 18	1 24	0 24	1 17	0 17	0 02
M entro	1 49	0 02	1 47	0 03	1 46	0 02	0 04
M-dmom1	3 81	0 24	3 56	0 35	3 46	0 26	0 02
M-dmom2	0 398	0 030	0 369	0 040	0 360	0 029	0 04
M-massec	0 472	0 006	0 474	0 009	0 464	0 006	0 03
S-rpc	0 0582	0 0030	0 0535	0 0082	0 0493	0 006	0 01
S-contr	0 491	0 023	0 441	0 091	0 391	0 056	0 02
S-dmom1	0 689	0 035	0 682	0 075	0 613	0 049	0 02
S-dmom2	0 0816	0 0037	0 0772	0 010	0 0696	0 0073	0 009

* p-value of the Terpstra trend test

Comparison of pCIN III and uCIN III

DNA ploidy patterns of the 29 uCIN III lesions of unknown progressive potential were diploid in one case (3%), polyploid in 15 (52%) cases and aneuploid in 13 (45%) cases (Table 2). When compared with the ploidy patterns in pCIN III lesions (26 % DNA-polyploid and 74 % DNA-aneuploid) the DNA-polyploidy in uCIN III lesions was more frequent. The mean 2 5c ER value of the uCIN III lesions was 89 %, which was similar to that of the pCIN III lesions. The mean 5c ER value of the uCIN III lesions was 26 %, that of the pCIN III lesions 31%. For the cluster analysis of pCIN III and uCIN III, at first those features were chosen, that were selected by the stepwise discriminant analysis (S-sdi, M- and S-gmrat, M- and S-circ and S-dmom1) With these features in the cluster analysis

no subdivision in clusters occurred. However, with the features which were selected in an earlier study, based on paraffin-embedded tissue, an indication of a subdivision in two clusters did occur (18). These variables included the standard deviation of a texture feature (S-homog), the standard deviation of a density feature (S-di), and the standard deviation (S-area) and mean value (M-circ) of two geometry features. The majority (91%) of pCIN III lesions was assigned to the same cluster (cluster 1). Of the uCIN III lesions 20 cases (69%) were assigned to this cluster 1. The 9 (31%) remaining uCIN III lesions were selected in the second cluster. The mean and standard deviation values of the clustering variables of the detected clusters are indicated in table 6.

Table 6: The mean values (and standard deviation values between brackets) of the clustering variables of the detected clusters.

	cluster 1		cluster 2	
S-homog	0.0738	(0.0033)	0.0805	(0.0055)
S-di	0.83	(0.14)	1.20	(0.16)
S-area	24.1	(3.8)	33.3	(3.8)
M-circ	0.824	(0.012)	0.820	(0.012)

A translation of these findings is that cluster 1 lesions have less variation in nuclear DNA-distribution, DNA-content and nuclear size, and have, on the average, more round nuclei. Translated into light microscopical terms these features could be descriptive of relatively small-rounded hyperchromatic nuclei, as seen in the relative uniform morphology of classical in situ lesions. Whereas a greater variability in size and chromatin distribution could be consistent with severely dysplastic changes.

DISCUSSION

The first aim of the present study was to describe DNA-ploidy patterns, 2.5c and 5c exceeding rates and cytophotometric features values of progressive pCIN lesions. The results indicated that, with increasing severity the pCIN lesions developed a higher rate of DNA-aneuploidy, ranging from 20% in pCIN I lesions, to 74% in pCIN III lesions. In parallel 2.5c exceeding rates increased from 66% in pCIN I lesions to 89% in pCIN III lesions. These findings linked up with previ-

ous data of Nasiell et al who showed that mild or moderate dysplastic lesions which progressed towards carcinoma in situ or microinvasive carcinoma, were invariably accompanied by a sequence of increasing abnormal DNA patterns (32). They further noted that in three patients with cervicitis as the final diagnosis, DNA patterns were constantly within normal limits. Our present results of DNA ploidy analysis and exceeding rates in individual patients with successive pCIN and INV lesions, however indicated that changes of DNA-aneuploidy into DNA-polyploidy did occur with increasing severity of the lesions. Furthermore the fact that DNA-polyploidy did occur in the group of progressive CIN lesions and in the group of invasive cancer lesions, is in conflict with previous suggestions that DNA-polyploidy could be considered as indicative for regression in CIN lesions (6,11). In the present study DNA-polyploidy was found in cervical cytologic smears in CIN lesions which were proven to be progressive and in invasive lesions. Therefore DNA-polyploid CIN lesions should not be regarded as possibly reactive or regressive.

The finding that DNA-diploidy did not occur in the present group of cytologic smears, from progressive CIN lesions and invasive cancer, might add to the idea that DNA-diploidy in cervical smears demonstrates non-progressive "CIN" lesions, as was suggested earlier (6,11,32). This seems contradictory to earlier findings of DNA-diploidy in CIN III lesions (1,2) and in CIN III-like lesions adjacent to invasive cancer in cytospin specimen retrieved from paraffin-embedded tissues (16). Recently, however, it was shown that DNA cytometric data and thus ploidy patterns are strongly dependent on the way the cells of interest are sampled, from cervical cytologic smears (CS) or retrieved from paraffin-embedded tissue by enzymatic digestion (PEC) (19). It was shown that 2.5c and 5c exceeding rates were significantly higher in the vast majority of cytologic specimens. DNA-diploidy was seen in four cases of CIN III in cytospin specimen made from paraffin-embedded tissue, while the corresponding cytologic smears showed polyploid and aneuploid DNA-patterns. These differences appeared to depend on the variance in selection of CIN III cells, using the CS or PEC sampling method. At light microscopical selection of cells from conventional cytologic smears it is understandable that the most abnormal looking cells are sampled by preference whereas in specimens made from selected areas from paraffin embedded tissue, all nuclei present in the cytospin specimen are accepted for analysis by the observer. Consequently cytophotometrical data from CS and PEC specimen from CIN III lesions can not be regarded to be interchangeable among each other (19).

The results of the high resolution cytophotometric analysis of the group of pCIN I-III and INV lesions showed that trend-like changes could be identified in lesions with increasing severity c.q. from pCIN I and pCIN II towards pCIN III and INV. The cyto-morphological translation of these findings was that with increasing atypia of the progressive lesions, nuclei tended to have less finely granular chromatin, less contrast between the chromatin particles, less variation in contrast between the chromatin particles, and more DNA margination. Greatest differences were detectable when pCIN I/pCIN II lesions were compared with pCIN III/INV lesions.

When comparing pCIN III and the uCIN III lesions, DNA-polyploidy occurred more often in uCIN III and the 5c exceeding rates were slightly lower in uCIN III, but differences regarding DNA ploidy patterns or the 2.5c or 5c exceeding rates were not significant. It was of interest that a cluster analysis performed on the basis of four photometric features that were previously used in the cluster analysis of CIN III and CIN III like lesions adjacent to invasive cancer, was applicable for the group of pCIN III and uCIN III (18). 69% of the uCIN III lesions were member of the same cluster 1, as the vast majority of the pCIN III lesions. The earlier high resolution cytophotometric study of CIN III and CIN.INV showed that 90% of CIN.INV lesions were member of one and the same cluster 1, while 56% of the CIN III lesions with unknown progressive potential had cluster 1 feature values. These different studies indicated as such that CIN III lesions with unknown progressive potential could be subdivided in two subgroups, using high resolution cytophotometric analysis. Adding to these findings the present study showed that by high resolution cytophotometric analysis, a trend-like pattern could be described in CIN lesions with a progressive course. It may be considered to apply a classifier based on a combination of age, high resolution cytophotometric data and DNA ploidy patterns, in a prospective study of cytologically detected CIN lesions.

It is argued that the profile of progressive cervical intraepithelial lesions has become more clear and that it might be justified to follow in time those lesions that do not come up to this profile, without the patient taking risks when not treating the non-progressive lesions.

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SUMMARY

The natural history of premalignant epithelial lesions of the uterine cervix has not been solved. Cervical intraepithelial neoplasia (= CIN I-III or dysplasia and carcinoma in situ) may have a progressive course towards invasive cancer, but they may also persist or return to normal. Cytopathologic, histopathologic or colposcopic investigations have not been able to subdivide these lesions into progressive, persistent and regressive subtypes. As a consequence all patients are presently treated as having a potentially progressive lesion.
A technique that could distinguish between potentially progressive and potentially persistent or regressive lesions would be of great value for the treatment of cervical abnormalities. The subject of the present study, was the significance of DNA cytometry of cervical intraepithelial neoplasia.

In chapter 1 an overview of some of the literature is given, with some extra attention for the role of human papilloma virus infection as a possible progression marker.
The aim of the study was to investigate the value of DNA cytometry for the discrimination between progressive and non-progressive cervical intraepithelial neoplasia.

In chapter 2 a method is described, which was developed to extract nuclei from circumscribed epithelial regions in 50 micrometer sections of paraffin-embedded tissue. The area of interest is isolated from these thick sections, by scraping off the non-relevant tissue with a scalpel under a dissection microscope. After extraction of the nuclei in a protease solution and washing, the nuclei can be used for flow cytometric analysis and for image cytometric analysis, after being resuspended in foetal calf serum and cytocentrifugation. As examples of the technique flow and image cytometric measurements are presented of nuclei isolated from a) carcinoma in situ region and invasive carcinoma region in breast tissue, and b) from cervical intraepithelial neoplasias grade 3 (CIN III).

The study, presented in chapter 3, reports on image (cytophotometric) and flow cytometric DNA ploidy analysis in nuclei isolated from selected CIN III areas of paraffin-embedded tissue of 20 patients with (CIN), and 19 patients without

(CIN.INV) synchronous cervical invasive squamous cell carcinoma. 21 patients were 35 years of age or younger and 18 patients were 50 years or older. DNA-aneuploidy was about as frequent in the complete group of CIN lesions (53%) as in the CIN.INV group (50%). Differences in the distribution of DNA ploidy patterns between the two age groups were observed. The majority of CIN III lesions in women 50 years of age or older were aneuploid. This was observed in CIN III lesions with (70%) as well as without (88%) a coexisting invasive cancer. In the group of younger women a diploid DNA pattern was found in about 60% of the CIN III-like lesions with a concomitant invasive cancer. In the absence of an invasive cancer, CIN III lesions were mostly polyploid. The DNA patterns of invasive cancer were generally identical with the adjacent CIN III-like lesions, thus suggesting the two lesions to be related. These results are at considerable variance with previously published data on DNA ploidy values in CIN lesions. As a consequence of these results the prognostic value of DNA ploidy measurements in cervical intraepithelial lesions in women of these two age groups had to be further evaluated. Parallel sections of all specimen were immunohistochemically stained for human papilloma virus capsid antigen (HPV). HPV could be demonstrated in four CIN III lesions without invasive cancer, in women 35 years of age or younger. In three of these a polyploid DNA pattern was present; the fourth case showed a bimodal aneuploid pattern.

In chapter 4 DNA ploidy analysis and high resolution cytophotometric analysis were performed in a group of 37 CIN III lesions and 32 cases of cervical invasive squamous cell carcinoma (INV). Ploidy analyses, performed in nuclei isolated from selected CIN III and invasive cancer areas of paraffin-embedded tissue, showed DNA-aneuploidy to be about as frequent in the total group of patients with CIN III lesions (41%) as in the total group of patients with invasive (37%) lesions. When the patients were subdivided in two age groups, e.g. 45 years of age or younger and 46 years or older a relatively high percentage of DNA-diploidy was demonstrated in invasive lesions in the group of younger patients (73%). In CIN III lesions in younger women, DNA-polyploidy was relatively frequent (46%). In elderly women CIN III lesions showed the highest percentages of DNA-aneuploidy (82%).

The results of the high resolution cytophotometric analysis, based on twenty-one photometric features describing geometrical, density and texture characteristics of the digitized nuclear images, indicated that the overall mean values of 16 nuclear features discriminated significantly between the complete groups of CIN III and

invasive lesions. Based on the results of a stepwise linear discriminant analysis of mean values on a patient basis, a combination of two geometrical and two runlength texture features was used to discriminate between CIN III and invasive lesions. The correct classification rate of the complete groups of CIN III and invasive lesions was 78% each. The correct classification rate of CIN III and invasive cancer was lowest in the younger age group, being 77% and 60% respectively, and highest in the group of older patients, being 91% and 94% respectively. The results of this study indicated that high resolution cytophotometric analysis is of value in the discrimination between CIN III and invasive cancer. It was suggested that this type of analysis may be of help in the study of progressive and non-progressive CIN lesions.

In chapter 5 the results are described of high resolution cytophotometric analysis of 57 cases of CIN III, retrieved from selected areas of paraffin-embedded tissues. 37 Cases of CIN III of patients without synchronous cervical invasive carcinoma were regarded to represent a mixture of progressive and non-progressive lesions. 20 cases of CIN III lesions of patients with a synchronous cervical invasive squamous cell carcinoma were regarded to represent truly progressive precursor lesions (CIN.INV). Twenty-one photometric features describing geometrical, density and texture characteristics were extracted from the digitized nuclear images. Mean (M) and standard deviation (S) values of the photometrical features were calculated for each specimen. Statistical analysis of the cytophotometric data indicated significant differences between the complete groups of true CIN III and CIN III-like lesions concomitant with invasive carcinoma. A cluster analysis, using one co-occurrency texture feature, one density feature and two geometrical features, identified two clusters (C1 and C2) in the combined group of CIN III and CIN.INV specimens. The vast majority of CIN.INV lesions belonged to cluster C1. The CIN III group appeared to be a mixture of the two clusters, 54 % C1 and 46 % C2 lesions. The presence of this subgroup of lesions in the group of CIN III lesions (cluster C1) with similar photometric feature values as found in the vast majority of CIN.INV lesions, could very well represent a group of potentially progressive lesions. Based on these findings, in total some 40-50% of CIN III lesions would be potentially progressive. In patients older than 45 years, the percentage of these potentially progressive CIN III lesions was 27%. In patients 45 years or younger the percentage of these potentially progressive CIN III lesions was 62%.

In chapter 6 a cytophotometric analysis was performed of cytological smears and corresponding cytospin specimen, retrieved from selected areas of paraffin-embedded tissues, representing Cervical Intraepithelial Neoplasia grade 3 lesions from 22 patients. The average time interval between cytological and histological diagnosis was six weeks. A visual classification of DNA ploidy patterns was performed, and the 2.5c and 5c exceeding rates were calculated. Furthermore a high resolution cytophotometric analysis was performed using the specimen mean and standard deviation values of 21 photometric features. A significant correlation existed between DNA ploidy patterns in corresponding cytospin specimens made from larger epithelial areas and conventional cytological smears. The 2.5c and 5c exceeding rates however were found to be significantly higher in the conventional cytological smears. Also at high resolution cytophotometric analysis of cell nuclei in cytological smears and cytospin specimens made from larger epithelial areas specimen, significant differences for a large number of nuclear photometric features were found. These findings might result from differences in the selection of CIN III cells from conventional cytological smears and cytospin specimens made from larger epithelial areas and by differences in fixation procedures as used for the two types of specimens. It is concluded that cytophotometric data of conventional cytological smears and cytospin specimens made from larger epithelial areas representing CIN III lesions should not be regarded as interchangeable among each other.

In chapter 7 DNA ploidy analysis, 2.5C and 5C exceeding rates and high resolution cytophotometric analysis were performed in 73 cervical cytological smears with cytologial diagnosis of CIN I, II, III and invasive carcinoma from 57 women, to identify prognostic parameters. From four cervical screening registries a group of 28 women was selected on basis of a final histologic diagnosis of cervical invasive squamous cell carcinoma, with previous cervical smears consistent with CIN I, II and III lesions and who were left untreated. A second group of 29 patients, who were treated for cytologically diagnosed CIN III was regarded to represent a mixture of progressive, persistent and regressive lesions. In 64% of cervical smears from the group of patients who had developed invasive cancer, a DNA-aneuploid pattern was found both in CIN and in invasive lesions. DNA polyploidy was present in 36% of both of these lesions. It was concluded that DNA polyploid CIN lesions should not be regarded as possibly reactive or regressive. DNA diploidy did not occur in these CIN or invasive cancer cases. The 2.5C exceeding rate increased with increasing severity of the CIN lesion. A high

resolution cytophotometric analysis demonstrated a trend-like pattern of a number of feature values in lesions with increasing severity, thus from CIN I and CIN II to CIN III and invasive cancer. In a cluster analysis based on the results of high resolution cytophotometric analysis, a subdivision of CIN III lesions with unknown malignant potential was possible into a group of lesions with similar feature values as a group of progressive CIN III and a second group of lesions that had feature values that were dissimilar to progressive CIN III. It was suggested to apply a classifier based on a combination of age, high resolution cytophotometric data and ploidy patterns in a prospective study of cytologically detected CIN-lesions.

SAMENVATTING

Het natuurlijke beloop van premaligne afwijkingen van de cervix uteri is nog niet bekend. Cervicale intra-epitheliale neoplasmata (= CIN of dysplasie en carcinoma in situ) kunnen een progressief gedrag vertonen en invasief worden, maar kunnen ook persisteren of verdwijnen. Het is niet mogelijk gebleken, op basis van cytopathologisch, histopathologisch of kolposkopisch onderzoek, deze laesies onder te verdelen in progressieve, persisterende en regressieve sub-types. Als gevolg hiervan worden patiënten met CIN laesies behandeld als betrof het in alle gevallen, potentieel progressieve laesies. Een techniek die bijdraagt aan het onderscheiden van laesies met een progressief karakter en laesies met een niet-progressief gedragen, is dringend gewenst. De studie, beschreven in dit proefschrift, betreft de toepassing van DNA cytometrische analyse op cervicale intra-epitheliale neoplasmata, met het doel kenmerken van celkernen te identificeren die een afspiegeling vormen van het biologische karakter van de afwijking.

In hoofdstuk 1 wordt een overzicht gegeven van relevante literatuur over het natuurlijk beloop van CIN laesies en over DNA cytogenetisch en cytometrisch onderzoek. Daarbij wordt enige extra aandacht geschonken aan de mogelijke betekenis van de detectie van humaan papilloma virus (HPV) infectie voor het onderscheid tussen progressieve en non-progressieve CIN laesies.

In hoofdstuk 2 wordt een methode beschreven die ontwikkeld werd om kernen te isoleren uit geselecteerde gebieden van in paraffine ingebed weefsel. Daartoe worden 50 micrometer dikke coupes gesneden, gedeparaffineerd en gerehydreerd. De "dikke" coupes worden op een glaasje gebracht waarna onder een dissectiemicroscoop het geselecteerde gebied kan worden geïsoleerd, door het overtollige weefsel met een scalpel te verwijderen. Een optimaal aantal kernen wordt verkregen na incubatie met protease-oplossing. Na wassen kunnen de kernen gebruikt worden voor flow-cytometrisch onderzoek. Na suspensie in foetaal calf-serum, cytocentrifuge en Thionine-Feulgen kleuring kunnen de kernen geanalyseerd worden m.b.v. beeld-analytisch cytometrisch onderzoek. De methode wordt

verduidelijkt met voorbeelden van een carcinoma in situ en invasief carcinoom van de mamma en een CIN III van de cervix uteri.

De studie zoals gepresenteerd in hoofdstuk 3 meldt de resultaten van image- en flow-cytometrische DNA ploidie analyse van kernen, geïsoleerd uit geselecteerde CIN III gebieden van in paraffine ingebed weefsel van 20 patiënten met (CIN), en 19 patiënten zonder (CIN.INV) synchroon invasief plaveiselcelcarcinoom van de cervix. 21 Patiënten waren 35 jaar of jonger en 18 patiënten waren 50 jaar of ouder. Een aneuploid DNA-patroon werd ongeveer even frequent gezien in de gehele groep van CIN laesies (53%) als in de groep van CIN.INV laesies (50%). Verschillen in de verdeling van de DNA ploidie patronen tussen de twee leeftijdsgroepen werden waargenomen. Het merendeel van CIN III laesies bij vrouwen 50 jaar of ouder was aneuploid, zowel met (70%) als zonder (88%) een gelijktijdig aanwezig invasief carcinoom. In de groep jongere vrouwen werd een diploid DNA patroon in ongeveer 60% van de CIN.INV laesies getroffen. Bij afwezigheid van een invasief carcinoom waren de meeste CIN III laesies polyploid. De DNA patronen van invasieve carcinomen kwamen in het algemeen overeen met die van het aangrenzende CIN.INV, suggererend dat de twee laesies aan elkaar verwant waren. Deze resultaten wijken nogal af van eerder gepubliceerde gegevens over DNA ploidie analyse bij CIN laesies. De prognostische waarde van DNA ploidie metingen bij cervicale intra-epitheliale afwijkingen bij vrouwen in deze twee leeftijdscategorieën werd daarom nader onderzocht. Parallelle coupes van alle preparaten werden immunohistochemisch gekleurd voor humaan papillomavirus kapsel antigeen. Het HPV virus kon aangetoond worden bij vier CIN laesies zonder invasief carcinoom bij de jongere vrouwen. In drie van deze gevallen was een DNA-polyploid patroon aanwezig; het vierde geval toonde een bimodaal aneuploid DNA-patroon.

In hoofdstuk 4 werd DNA ploidie analyse en hoge resolutie cytofotometrische analyse verricht in een groep van 37 CIN III laesies en 32 gevallen van cervicaal invasief plaveiselcelcarcinoom (INV). De patiënten werden onderverdeeld in twee leeftijdsgroepen, nl. 45 jaar of jonger en 46 jaar of ouder. De DNA-ploidie analyse, verricht op kernen geïsoleerd uit geselecteerde CIN III - en INV gebieden van paraffine ingebed materiaal, toonden dat DNA aneuploidie ongeveer even frequent was in de totale groep van CIN III laesies (41%) als in de totale groep van INV (37%) laesies. Een relatief hoog percentage DNA diploidie werd aangetroffen in invasieve laesies bij jongere patiënten (73%). DNA polyploidie was relatief frequent (46%) bij CIN III laesies bij jongere vrouwen. Bij oudere

vrouwen met CIN III laesies was DNA aneuploidie zeer frequent (82%). Hoge - resolutie cytofotometrische analyse, van gedigitaliseerde kernen, gaf aan dat de gemiddelde waarden van 16 kern-kenmerken een significant onderscheid maakten tussen de gehele groep CIN III en INV. Op basis van de resultaten van een stapsgewijze lineaire discriminant-analyse van de gemiddelde waarden per patiënte, werd de combinatie van twee geometrische en twee textuur-kenmerken gebruikt om CIN III en INV laesies te onderscheiden. De groepen van CIN III en INV laesies werden juist geclassificeerd in 78%. De juiste classificatie van CIN III en INV was het laagst in de jonge leeftijdsgroep, 77% en 60% respectievelijk, en het hoogst in de groep van oudere patiënten, 91% en 94% respectievelijk. De resultaten van deze studie geven aan dat hoge resolutie cytofotometrische analyse van betekenis kan zijn voor het onderscheid tussen CIN III en invasief carcinoom, en mogelijk bij de studie van progressieve en niet-progressieve CIN laesies.

In hoofdstuk 5 wordt een studie beschreven van hoge resolutie cytofotometrische analyse van 57 gevallen van CIN III, afkomstig van geselecteerde gebieden van in paraffine ingebed weefsel. 37 Gevallen van CIN III van patiënten zonder gelijktijdig invasief carcinoom van de cervix, werden beschouwd als een verzameling van progressieve en niet-progressieve laesies. Van 20 gevallen van CIN III laesies bij patiënten met een synchroon cervicaal invasief plaveiselcelcarcinoom, werd aangenomen dat dit daadwerkelijk progressieve afwijkingen (CIN.INV) waren. Per preparaat werden de gemiddelde en standaard deviatie waarden van 21 fotometrische kenmerken m.b.t. geometrische, dichtheids- en chromatine textuur karakteristieken, berekend uit de gedigitaliseerde kernbeelden. Statistische analyse van de cytofotometrische gegevens gaf significante verschillen aan tussen de complete groepen CIN III en CIN.INV. Cluster-analyse, gebruikmakend van een chromatine-textuurkenmerk, een dichtheidskenmerk en twee geometrische kenmerken, toonde twee clusters (C1 en C2) aan in de gehele groep van CIN III en CIN.INV preparaten. Het overgrote deel van de CIN.INV afwijkingen behoorde tot een en dezelfde cluster C1. De CIN III groep bleek te bestaan uit een mengsel van de twee clusters, 54% C1- en 46% C2-laesies. De aanwezigheid van een subgroep in de CIN III groep met overeenkomstige fotometrische waarden als de overgrote meerderheid van CIN.INV laesies, zou kunnen duiden op een progressieve potentie van deze subgroep. Als deze bevindingen bevestigd worden, zou ongeveer 40-50% van de CIN III laesies potentieel progressief zijn. Bij patienten ouder dan 45 jaar was dit percentage 27%. Bij patiënten 45 jaar en jonger was dit percentage 62%.

In hoofdstuk 6 wordt een cytofotometrische analyse beschreven van CIN III laesies van 22 patiënten met corresponderende cytologische uitstrijkpreparaten en cytospin-preparaten, opgewerkt van geselecteerde gebieden van in paraffine ingebed weefsel. De gemiddelde interval-periode tussen de cytologische- en histologische materiaal-afname was 6 weken. De DNA ploidie patronen en de 2.5C en 5C exceeding rates werden beoordeeld. Verder werd een hoge resolutie cytofotometrische analyse verricht, gebruik makend van de gemiddelde en standaard-deviatie waarde van 21 fotometrische kern kenmerken. Een significante correlatie was aanwezig tussen de DNA ploidie patronen in de corresponderende paraffinecytospin- en uitstrijk-preparaten. De 2.5c en 5c exceeding rates waren echter duidelijk hoger in de uitstrijkpreparaten. De hoge resolutie cytofotometrische analyse van celkernen in uitstrijk- en paraffinecytospin-preparaten toonde significante verschillen voor een groot aantal fotometrische kern-kenmerken aan. Deze bevindingen kunnen mogelijk verklaard worden door verschillen in selectie van CIN III cellen in uitstrijk- en paraffinecytospin-preparaten en door verschillen in fixatie-procedures, zoals gebruikt voor de twee technieken. Geconcludeerd wordt dat cytofotometrische gegevens van uitstrijk- en paraffinecytospin-preparaten van CIN III laesies niet zonder meer vergelijkbaar zijn.

Hoofdstuk 7 maakt melding van de toepassing van DNA ploidie analyse, 2.5C en 5C exceeding rate berekeningen en hoge resolutie cytofotometrische analyse in 73 uitstrijkpreparaten van 57 vrouwen. Uit vier cervix screening registraties werd een groep van 28 vrouwen geselecteerd, die na een eerdere cervix uitstrijk, passend bij CIN I, II en III laesies, uiteindelijk een invasief carcinoom hadden ontwikkeld. Een tweede groep van 29 patiënten werd geselecteerd die behandeld was in verband met de cytologische diagnose CIN III. Van deze groep werd verondersteld dat het een menggroep was van progressieve, persisterende en regressieve laesies.

In 64% van de cervix uitstrijken van de patiënten die uiteindelijk kanker hadden ontwikkeld, werd een DNA aneuploid patroon gevonden, zowel in de CIN als in de invasieve laesies. DNA polyploidie was aanwezig in 36% van deze laesies. Geconcludeerd werd, dat DNA polyploide CIN laesies niet beschouwd kunnen worden als mogelijk reactief of regressief. In deze gevallen van progressieve CIN laesies en invasieve carcinomen werd geen DNA diploidie aangetroffen.

Met het toenemen van de ernst van de CIN laesie nam de 2.5C exceeding rate toe. De hoge resolutie cytophotometrische analyse gaf aan dat voor een aantal parameters een trend te ontdekken was die parallel liep met toenemen van de

ernst van de laesie, c.q. van CIN I, CIN II en CIN III naar invasief carcinoom. Een cluster analyse, gebaseerd op de resultaten van de hoge resolutie cytophotometrische analyse gaf aan dat de groep van CIN III laesies met een onbekend progressief potentieel onder te verdelen was in een groep die overeenkomstige kenmerken had als de groep van progressieve CIN III laesies en een tweede groep met waarden die niet pasten bij de progressieve CIN III laesies. De huidige resultaten rechtvaardigen het starten van een prospectieve studie van CIN laesies in cervix uitstrijken.

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DANKWOORD

DANKWOORD

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CURRICULUM VITAE

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 20 maart 1953 geboren te Tilburg. Na de Middelbare Schoolopleiding (HBS-B) te Tilburg, werd de studie Geneeskunde aan de Katholieke Universiteit Nijmegen in 1971 begonnen. Het artsexamen werd in 1980 behaald. Van 1973 tot 1976 was hij als docent Anatomie/Fysiologie werkzaam te Cuyk. Van 1980 tot 1981 vervulde hij de militaire dienstplicht. In 1981 was hij gedurende een korte periode als gast-medewerker verbonden aan de afdeling Farmacologie van de Katholieke Universiteit Nijmegen (hoofd: Prof. Dr. E.J. Ariens), waarna in datzelfde jaar een aanstelling volgde als wetenschappelijk medewerker op de afdeling Pathologie van de Katholieke Universiteit Nijmegen (hoofd: Prof. Dr. G.P. Vooijs). Van 1982 t.e.m. 1986 volgde hij de opleiding tot patholoog anatoom aan de Katholieke Universiteit Nijmegen (opleiders: Prof. Dr. P.H.M. Schillings en Prof. Dr. G.P. Vooijs). In 1984 trouwde hij met Ellen van Loevezijn. In 1985 werd de Centrale Nederlandse Registratie van clear cell carcinoom van vagina en cervix opgericht. In 1986 werd hij aangesteld als patholoog in het Academisch Ziekenhuis Nijmegen als consulent voor de gynaecologische pathologie, de cytopathologie en de kwantitatieve microscopie. In 1986 volgde verhuizing naar Den Haag, alwaar in 1987 Caspar en in 1989 Rogier werden geboren. Van 1986 t.e.m. 1988 was de schrijver van dit proefschrift lid van de ovariumtumorencommissie van het Koningin Wilhelmina Fonds, in 1988 gevolgd door het secretarisschap van de Landelijke Werkgroep Gynaecologische Pathologie. In 1990 werd hem een NWO/NAVO stipendium toegekend voor onderzoek in de University of California in San Francisco naar kwantitatief microscopische, immunohistochemische en hybridocytochemische kenmerken van borderline maligne ovariumtumoren.

STELLINGEN

behorende bij het proefschrift

"DNA cytometry of

cervical intraepithelial neoplasia"

A.G.J.M. Hanselaar

I

Ten hoogste een op de tien premaligne cervix laesies zal, indien onbehandeld, uiteindelijk ontaarden in een invasief plaveiselcelcarcinoom. L.G. Koss, JAMA 261 5:737-743, 1989.

II

DNA-polyploidie is een frekwente bevinding bij progressieve premaligne cervix laesies en dient dus niet beschouwd te worden als indicatief voor een regressief gedrag.

III

Resultaten van DNA-cytometrisch onderzoek van cytologische uitstrijkpreparaten en van paraffine-ingebed materiaal zijn niet zonder meer onderling vergelijkbaar.

IV

DNA cytometrie kan, mits toegepast onder stringente condities, waardevol blijken bij screening van cervix laesies.

V

Borderline maligne ovariumtumoren met immunohistochemisch gedetecteerde, infiltrerende tumorcellen zijn carcinomen.

VI

Kankertherapie maakt een ontwikkeling door waarbij de specificiteit voor tumor en patiënt toeneemt. Kanker is als onkruid in een fraaie tuin; het onkruid moet gewied worden, de tuin behouden blijven. Adrian Linden, 1985.

VII

Huisartsen van patiënten met een maligne proces dienen betrokken te zijn bij multidisciplinaire oncologie-besprekingen. Dramatische misinterpretaties van persoonlijkheid en thuissituatie van patiënten kunnen zo voorkomen worden.

VIII

Patiënt-gebonden wetenschappelijk onderzoek in de pathologie is niet meer denkbaar zonder de PALGA.

IX

Evaluatie van wetenschappelijk onderzoek dient te geschieden aan de hand van publikaties in wetenschappelijke tijdschriften, zeker indien dit (mede-)gefinancierd wordt met overheidsgelden. Het is overigens de vraag of de citatie-index hiervoor het meest geëigend is.

X

Medisch specialisten dienen niet slechts een adviserende, maar een (mede-) beslissende stem te hebben in het ziekenhuis management.

XI

Sport als ontspanning draagt bij aan een gezond lichaam en een gezonde geest. "Top"sport doet dat veelal niet.

XII

Baliepersoneel van PTT en NS wedijveren om de hoogste klant-onvriendelijkheid. Vooralsnog onbeslist.

XIII

Het is absurd dat auto's nog steeds aangedreven worden door een zelfde type milieubelastende verbrandingsmotor als 100 jaar geleden.

Nijmegen, 23 oktober 1990